INSIGHT ON MECHANISMS OF OSMOTIC TOLERANCE

A Dissertation

by

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ABSTRACT

Industrial biotechnology provides a sustainable and environmentally sound way to produce chemicals by using renewable raw materials. Sustainability of industrial biotechnology can be further improved if alternative water sources such seawater and wastewater can be utilized instead of freshwater in fermentation. However, one of the challenges in the microbial production of chemicals is the low tolerance of the host to inhibitors present in the feedstocks and the alternative water sources, which inhibits the widespread of their usage. Thus, strain development with desired phenotypes is needed to expand the utilization of the renewable feedstocks and the alternative water sources. However, poor understanding of the mechanisms for most complex traits, such as tolerance to inhibitors, pose a major challenge to rational strain development for more robust host producers.

In this dissertation, a focus is made on developing microbial hosts with increased tolerance to osmotic stress, specifically sodium chloride (NaCl). This dissertation begins with the validation of the osmotic tolerance of an *rpoC* mutation which has been identified in separately evolved osmotolerant mutants of *Escherichia coli*. Using a combination of single amino acid supplementation, metabolites quantification, membrane stability and global transcriptional analysis, we found that the osmotic tolerance conferred by this *rpoC* mutation may be related with higher production of acetic acids and amino acids, increased membrane integrity, upregulation of genes *metK* and *mmuP*, and downregulation of a stress related gene *bolA*. The next section describes

the utilization of seawater to improve the production of carotenoids in *Saccharomyces cerevisiae*. The NaCl supplementation tests and lipid analysis demonstrated that the carotenoids production improvement in seawater is partially related with additional NaCl presented in seawater and may also related with lipid content and composition. The following section describes the improvement of osmotic tolerance and growth of *Saccharomyces cerevisiae* strain SM14 in seawater via adaptive laboratory evolution, and we identified mutations in *WHI2* that confer the improved osmotic tolerance in SM14. In conclusion, these findings expanded our current understanding of osmotic tolerance in *E. coli* and *S. cerevisiae*, and have the potential to expand the utilization of high saline feedstocks and water sources in microbial fermentation.

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1 INTRODUCTION

Industrial biotechnology, which is also called white biotechnology, is a promising field for chemical production. By using biological systems and renewable raw material, fuels and chemicals can be produced in a more sustainable and environmentally friendly manner. The performance of the microbial hosts used for biochemical conversion is a key factor for the choice of feedstock and method for product separation, which greatly affect the production cost of a process. One of the challenges in the microbial production of chemicals is the low tolerance of the host to inhibitors present in the feedstocks or products. Thus, successful development of more robust producers is needed to improve productivity and reduce cost in a process. However, the genetic determinants underlying most complex traits of the hosts desired in industry are not well understood, which limits our ability to rationally design microbial hosts. Adaptive laboratory evolution, which is based on the Darwinian principle of natural selection, does not require a *priori* knowledge of genetic targets and molecular mechanisms of desired phenotypes, and thus is a powerful technique for strain development. During the propagation of microorganisms, mutations randomly occur, and the portion of individuals with beneficial mutations which confer fitness advantages over others will increase and finally become dominant in the population. By combining with other tools (*e.g.* whole-genome sequencing (1, 2), transcriptomics (3, 4), proteomics (5), and metabolomics (6, 7)), the genes and mechanisms involved in the mutants with fitness

advantages can be identified, which provides insights into the phenotypes of interest and enhances the knowledge underlying the complex phenotypes needed for the rational engineering of strains.

There is an increasing interest in using sustainable feedstocks such as lignocellulosic hydrolysates (8-10) and waste glycerol (11, 12). Due to water scarcity, there is also an increasing interest in using alternative water sources such as seawater and wastewater in fermentation. However, some of these feedstocks and water sources contain inhibitory components such as high salts that can negatively impact the performance of the microbial host, leading to reduced productivity (13-16). Thus, osmotic tolerance of microbial systems need to be improved to increase their productivity and expand the utilization of high saline feedstocks and water sources.

1.1 Osmotic stress

Osmotic stress is a sudden change of the solute concentrations in the external environment surrounding a cell. Hyperosmotic stress, which is an increasing of extracellular osmolality, caused by high concentration of either salts, substrates or other solutes, results in water efflux and shrinkage of cells; while hypo-osmotic stress, a decreasing of extracellular osmolality, causes water influx and swelling or even lysis of cells.

Osmotic stress perturbs many cellular properties. In *E. coli*, it has been reported that hyperosmotic stress severely inhibited active transport of carbohydrates, and this inhibition of transport was sufficient to cause complete inhibition of growth observed during severe osmotic upshift (17). Hyperosmotic stress also decreases cell volume and

the initiation frequency of DNA replication, inhibits cellular division, changes membrane and metabolite composition, and affects the stability and function of proteins (18-25). In *S. cerevisiae*, hyperosmotic stress also causes a reduction of cell volume, inhibits nutrition uptake, disturbs metabolite concentration, perturbs membrane composition, gene expression and cell division, and reduces fermentation activity (26-35).

1.2 Response and adaptation to hyperosmotic stress

The cellular response and adaptation to hyperosmotic stress have been extensively studied in bacteria. In *E. coli*, most transcriptional activities are rapidly altered in response to hyperosmotic stress (36-39). The global transcriptional response to osmotic upshift in *E. coli* was hypothesized to occur in four phases (40). The first phase is the inhibition of ribosomal RNA transcription (37), and the second phase includes the selective activation of genes such as transporters of osmoprotectants, and the production of σ^{38}, which regulates general stress response (41). In the third phase, σ^{38} directs transcription of genes confer stress resistance (42), and the final phase is the reversion or attenuating of these changes as the cell becomes fully adapted to the osmotic challenge (40).

The repression of ribosomal RNA transcription and the activation of the σ^{38} directed transcription are triggered by the accumulation of K^+ glutamate (40, 43). *E. coli* accumulates K^+ glutamate as a first response to hyperosmotic stress (44-46). There are three K^+ uptake systems (Trk, Kdp, and Kup) in *E. coli* and other bacteria such as *Salmonella* (40). Trk is the predominant K^+ uptake system; it is composed of a

membrane-integral K^+/H^+ symporter module, which is present as two separate homologous polypeptides, TrkG and TrkH (47). It also contains two peripheral, cytoplasmic proteins, TrkA and TrkE, which have NAD^+- and ATP-binding properties, respectively (48). The K^+ transport rate of Trk is the highest among the three K^+ uptake systems and only the K^+ influx of Trk will be stimulated by hyperosmotic stress while the efflux of K^+ remains constant (40). Thus, the influx of K^+ increases immediately upon osmotic upshift (47), and when the cells attain a new steady state, the influx rate of K^+ will decrease to the same level as the efflux rate (49). The second K^+ uptake system is Kdp system, which contains subunits encoded by the *kdpFABC* operon (50, 51). The expression of *kdpFABC* is regulate by the KdpD/KdpE two-component system (52). The sensor kinase KdpD responds to K^+ limitation and salt stress and the response regulator KdpE targets the *kdpFABC* operon (53). Comparing with Trk, the induction level of the kdpFABC complex imposed by NaCl is much lower (50, 51). The third K^+ uptake system is Kup, it serves as the dominant K^+ uptake system upon hyperosmotic stress induced by sugar at low pH values and nonlimiting K^+ concentrations, but does not respond to elevated NaCl concentrations (54). In addition to K^+, another charged solute, glutamate, also accumulates in *E. coli* in response to hyperosmotic stress, and this glutamate accumulation has been reported to be stimulated by the alkalization of the cytoplasm resulting from K^+ accumulation (40, 46, 55).

As a first response to cellular dehydration, the accumulation of K^+ glutamate only partially rehydrates the cells, but cannot restore growth to the pre-stress rate (56). Indeed, organic osmoregulatory solutes, which are able to restore cell hydration and

growth more effectively upon hyperosmotic challenge, can be accumulated in *E. coli* via *de novo* biosynthesis (*e.g.* trehalose) or uptake from environment (*e.g.* glycine betaine). In the absence of external osmoprotectants, *E. coli* synthesizes trehalose from UDP-glucose and glucose 6-phosphate by trehalose-6-phosphate synthase (OtsA) and trehalose-6-phosphate phosphatase (OtsB) (55). If osmoprotectants are available externally, *E. coli* can import them from environment. There are four transporters that mediate osmoprotectants uptake, namely ProP, ProU, BetT and BetU. ProP and ProU are ubiquitously exist in *E. coli*, and they mediate the uptake of proline, glycine betaine, proline betaine, ectoine, and other zwitterionic compounds (57, 58). ProP can be activated by low osmolality, while proU is only expressed at high osmolality (36, 39-41). Different from ProP and ProU, which are broad in substrates specificity, BetT is specific to the uptake of choline, which is the substrate to produce glycine betaine by BetA and BetB (59), while BetU, which specifically mediates uptake of betaines, only exists in some *E. coli* strains (60).

In addition to the regulation of intracellular osmolality, *E. coli* has been found to accumulate ubiquinone-8, which has been shown to improve stability of artificial liposomes when challenged with sustained hyperosmotic stress; the Δ*ubiG* deletion mutant showed significantly decreased osmotic tolerance compared with wild-type strain, suggesting that *E. coli* may withstand osmotic stress by accumulating ubiquinone-8 to enhance its cytoplasmic membrane stability (22).

In yeast, the responses to hyperosmotic challenge are mainly governed by the high osmolarity glycerol (HOG) signaling pathway (61). HOG includes two upstream

signaling pathways, Sln1 and Sho1. The Sln1 pathway is a two-component signal transduction system which is composed of a sensor histidine kinase and a response regulator. Under normal osmotic conditions, the activity of Hog1 is constitutively inhibited, as the Sln1 histidine kinase keeps active and phosphorylates the histidine phosphotransferases protein Ypd1 (62, 63), which constitutively phosphorylate Ssk1 (64, 65). Upon hyperosmotic challenge, the Sln1 histidine kinase will be inactivated, thus, the unphosphorylated Ssk1 will accumulate, bind and activate Ssk2/Ssk22 (66). The activated Ssk2/Ssk22 phosphorylates and activates Pbs2, which then activates Hog1 (67, 68). The other branch of the HOG pathway is Sho1, which also regulates the activation of Pbs2 and Hog1, however, its activation mechanism remains unclear (61). Once activated, Hog1 will rapidly accumulate in the nucleus to phosphorylate its nucleus substrates, including transcription factors and cell-cycle regulators, after a new osmotic homeostasis is established, it will be exported back to the cytoplasm (69, 70). The downstream responses of HOG include the accumulation of the compatible osmolytes, regulation of gene expression and cell-cycle progression (61). In *S. cerevisiae*, glycerol is currently known as the sole compatible solute upon osmotic upshift (34). The Hog1 MAPK pathway regulates the accumulation of glycerol by regulating its metabolic flux and transport (71-73). Ion import/export is also regulated by Hog1 via the phosphorylation of the Nha1 Na+/H+ antiport and the Tok1 potassium channel as an rapid initial relief of the stressed condition (74). Hog1 controls gene expression by phosphorylating transcription factors and associating with chromatin via physical interactions with the transcription factors (61). The active Hog1 also induces a delay of

cell cycle, which allows the cell to adapt to the stressed conditions before enter the next growth phase (75, 76).

1.3 Efforts to enhance osmotic tolerance

With the knowledge of response and adaption to osmotic stress, several efforts have been done to improve osmotic tolerance of microorganisms. For example, trehalose has been overproduced and resulted in a growth increase of *E. coli* in the presence of a variety of osmotic-stress agents (hexose sugars, inorganic salts, and pyruvate) (77). In *S. cerevisiae*, the HAL1 gene has been overexpressed to improve the tolerance of *S. cerevisiae* to NaCl by increasing intracellular K^+ and decreasing intracellular Na^+ (78). However, the improvement of osmotic tolerance by rational engineering has been limited due the limited knowledge of the genetic determinants and mechanisms involved. Thus, evolutionary engineering, which does not require prior knowledge on genetic targets, is an extremely useful tool for strain development with desired complex phenotypes. For example, our lab has successfully evolved *E. coli* for the ability to grow in up to 0.8 M NaCl in minimal media, while this condition completely inhibits the parental strain (79). Adaptive evolution has also been applied to significantly improve the fitness of *S. cerevisiae* under osmotic stress (80).

1.4 Objectives

The overall goal of this dissertation is to expand current knowledge on osmotic tolerance in microbial hosts.

Objectives of this dissertation include:

1. Elucidate the osmotic tolerance mechanisms conferred by a *rpoC* mutation identified from separately evolved osmotolerance mutants of *Escherichia coli.*
2. Improve carotenoids production of *Saccharomyces cerevisiae* in seawater.
3. Improve growth of *Saccharomyces cerevisiae* in seawater.

2 INSIGHTS ON OSMOTIC TOLERANCE MECHANISMS IN *ESCHERICHIA COLI* GAINED FROM AN *RPOC* MUTATION*

2.1 Summary

An 84 bp in-frame duplication (K370_A396dup) within the rpoC subunit of RNA polymerase was found in two independent mutants selected during an adaptive laboratory evolution experiment under osmotic stress in *Escherichia coli*, suggesting that this mutation confers improved osmotic tolerance. To determine the role this mutation in *rpoC* plays in osmotic tolerance, we reconstructed the mutation in BW25113, and found it to confer improved tolerance to hyperosmotic stress. Metabolite analysis, exogenous supplementation assays, and cell membrane damage analysis suggest that the mechanism of improved osmotic tolerance by this *rpoC* mutation may be related with higher production of acetic acids and amino acids such as proline, and increased membrane integrity in the presence of NaCl stress in exponential phase cells. Transcriptional analysis led to the findings that the overexpression of methionine related genes *metK* and *mmuP* improved osmotic tolerance in BW25113. Furthermore, deletion of a stress related gene *bolA* was found to confer enhanced osmotic tolerance in BW25113 and MG1655. These findings expand our current understanding of osmotic tolerance in *E. coli*, and have the potential to expand the utilization of high saline feedstocks and water sources in microbial fermentation.

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2.2 Introduction

One of the challenges in the microbial production of chemicals is the low tolerance of the microbial host to inhibitors present in the feedstock. There is increasing interest in using more sustainable feedstocks such as lignocellulosic hydrolysates (8-10) and waste glycerol (11, 12). However, some of these feedstocks contain inhibitory components such as high salts that can negatively impact the performance of the microbial host, leading to reduced productivity (13, 14). In addition, the use of seawater or wastewater as a replacement for freshwater in fermentation can help to alleviate freshwater demand in industrial biotechnology. However, partly due to the high salinity of these water sources and low osmotic tolerance of microbial hosts (15, 16), their adoption has not been widespread. Thus, improved osmotic tolerance in microbial systems can potentially increase their productivity when using high saline feedstocks, and expand the utilization of these alternate feedstocks and water sources.

As with other complex phenotypes, osmotic tolerance involves multiple genes and mechanisms. Existing studies have identified several cellular responses to osmotic stress in *Escherichia coli*. In response to increased osmotic pressure, the synthesis of aquaporin increases to accelerate the export of water to balance intracellular and environmental osmolality (81), the intracellular potassium concentration increases via the regulation of potassium transporters (82, 83), and the accumulation of osmoprotectants (*e.g.* trehalose, glycine betaine, proline, etc.) also increases (84-87). In addition to the regulation of intracellular osmolality, *E. coli* has been found to counter osmotic stress by accumulating ubiquinone-8 to enhance its cytoplasmic membrane

stability (22). Based on existing knowledge, there have been prior attempts to rationally improve osmotolerance in *E. coli* (77, 88). However, the levels of improvement (89-92) achieved have been modest, potentially due to limited knowledge of the genetic determinants and mechanisms involved.

We had previously identified an 84 bp in-frame duplication (K370_A396dup) in the RNA polymerization domain of RpoC of the RNA polymerase in two independently evolved osmotolerant mutants (79), which suggests that this *rpoC* mutation confers osmotic tolerance and warrants further study. We reconstructed this *rpoC* mutation in a wild-type BW25113 background and confirmed the beneficial effect of this mutation on osmotic tolerance. The reconstructed *rpoC* mutant exhibited improved growth in minimal media under osmotic stress compared with the wild-type strain. Since the *rpoC* mutation was selected during evolution in media supplemented with tryptophan (79), the impact of tryptophan supplementation was also investigated and found to confer increased osmotic tolerance, which has not been reported previously. Subsequent metabolite analysis, membrane damage analysis and transcriptional analysis of the mutant revealed potential mechanisms of how this specific *rpoC* mutation confers tolerance to osmotic stress.

2.3 Materials and methods

2.3.1 Media and growth conditions

M9 minimal media (per liter: 12.8 g $Na_2HPO_4 \cdot 7H_2O$, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 10 mg $FeCl_3 \cdot 6H_2O$, 1.8 mg $ZnSO_4 \cdot 7H_2O$, 1.2 mg $CuCl_2 \cdot 2H_2O$, 1.2 mg $MnSO_4 \cdot H_2O$, 1.8 mg $CoCl_2 \cdot 6H_2O$) supplemented with 0.5% (w/v) glucose and Luria-

Bertani (LB) broth were used for routine cultivation and growth assays. LB agar plates were used for strain isolation. Tryptophan (50 µg ml^{-1}), kanamycin (10 µg ml^{-1}), ampicillin (30 µg ml^{-1}) and chloramphenicol (25 µg ml^{-1}) were supplemented whenever necessary. All liquid cultures were cultivated with agitation at 37°C. Sodium chloride (NaCl) was utilized to adjust the osmotic strength of the media for growth assays. The starting pH of all the media used in this work was ~ 7.

2.3.2 Marker-less reconstruction of rpoC mutation in BW25113

In order to construct a marker-less *rpoC* K370_A396dup mutation in the wild-type BW25113 strain (F-, Δ*(araD-araB)567*, *lacZ4787(del)::rrnB-3*, *λ-*, *rph-1*, Δ*(rhaD-rhaB)568*, *hsdR514*), an Δ*argE744::kan* cassette, which resides 30,503 bp away from the *rpoC* gene in the chromosome, from JW3929 of the Keio collection (93) was transduced via P1 transduction (94) into the evolved osmotolerant mutant G3 (79) containing the *rpoC* K370_A396dup mutation. The transductants with Δ*argE744::kan* cassette were selected on LB agar plate with 10 µg ml^{-1} kanamycin. The kanamycin-resistant transductant containing the mutated *rpoC* allele was verified by colony PCR. The primers used for the colony PCR verification are: GAA ACC AAC TCC GAA ACC AA (forward) and AGT ACC GGT TCA AAT GCC TG (reverse). The transductant containing both the Δ*argE744::kan* cassette and the mutated *rpoC* allele was named EJW1. The Δ*argE744::kan-rpoC* K370_A396dup construct from EJW1 was then transduced into BW25113 selected for kanamycin resistance, and the same colony PCR screening procedure was used to isolate transductant EJW2, which contains both Δ*argE744::kan* and *rpoC* K370_A396dup. Then EJW2 was made arginine prototrophic

(arg+) via P1 transduction with the wild-type *argE* allele from BW25113 and selected on M9 minimal agar for arginine prototroph. The arg+ colonies that contain the *rpoC* K370_A396dup mutation was verified using the same colony PCR screening procedure. The resulting reconstructed mutant EJW3 therefore contains only the *rpoC* mutation from mutant G3 without any added markers. Since the original G3 mutant contained Δ*trpB769::kan* deletion, EJW3 was transduced with Δ*trpB769::kan* cassette from JW1253 of the Keio collection and selected on LB agar plate with 10 μg ml^{-1} kanamycin to create a tryptophan auxotroph, EJW4, similar to the one in the original G3 mutant. The same method was used to introduce this *rpoC* mutation in strain MG1655 (F-, *λ*-, *rph-1*), and the resulting MG1655 *rpoC* mutant was named EYG1. All strains used in this study are listed in Table 2.1.

Table 2.1 List of strains used.

Strains	Description/genotype	Source
BW25113	F-, Δ*(araD-araB)567, lacZ4787(del)::rrnB-3*, *λ*-, *rph-1*, Δ*(rhaD-rhaB)568, hsdR514*	CGSC
MG655	F-, *λ*-, *rph-1*	CGSC
Hfr-2×SFX-	BW25113 Δ*mbhA::oriT*, Δ*hyfC::oriT*, *trp::F[ΔtraST]*, (*gen*^{R}), parental strain of G3	(79)
G3	Evolved mutant of Hfr-2×SFX- containing *rpoC* K370_A396dup mutation	(79)
JW3929	BW25113 Δ*argE744::kan*	(93)
JW1253	BW25113 Δ*trpB769::kan*	(93)
EJW1	G3 Δ*argE744::kan*	This study
EJW2	BW25113 Δ*argE744::kan*, *rpoC* K370_A396dup	This study
EJW3	BW25113 *rpoC* K370_A396dup	This study
EJW4	BW25113 Δ*trpB769::kan*, *rpoC* K370_A396dup	This study
EYG1	MG1655 *rpoC* K370_A396dup	This study

2.3.3 Growth kinetic analysis

Frozen stocks of strains were streaked on LB agar plates for single colonies and incubated overnight at 37°C. Single colonies were used to inoculate 5 ml of fresh M9 media and incubated overnight at 37°C with shaking (225 rpm). The overnight cultures were washed once with fresh M9 media and resuspended in M9 to an OD_{600} of ~10.0, then 50 µl samples were inoculated in 5 ml M9 media supplemented with 0.6 M NaCl in 16 × 150 mm screw-capped test tubes, at an initial OD_{600} of approximately 0.1. The media was supplemented with 50 µg ml^{-1} tryptophan when necessary. Samples were incubated at 37°C with shaking (225 rpm), and growth was tracked by measuring the OD_{600} periodically using a spectrophotometer (Biomate 3, Thermo Scientific).

For viability assays, samples were plated from cultures sampled at different growth phases on LB plates. After overnight incubation at 37°C, colony forming units (CFU) were counted to estimate the concentration of viable cells. The size and shape of cells at different growth phases in M9 and M9 supplemented with 0.6 M NaCl were observed under a light microscope (Zeiss).

The ability of each strain to grow under higher osmotic stress was analyzed as described previously with an initial OD_{600} of ~0.05 and agitated at 275 rpm (a higher agitation was used to prevent cell clumping in the presence of higher osmotic stress). Screw-capped test tubes (16 × 150 mm) were used to analyze cell growth under micro-aerobic condition in M9 with addition of 0.8 M, 0.9 M, or 1 M NaCl. Normal test tubes (16 × 150 mm) were used to analyze cell growth under aerobic condition in M9 with

addition of 0.75 M, 0.8 M, or 0.9 M NaCl. Three biological replicates were used in each condition.

2.3.4 Effects of amino acid supplementation

The effects of individual amino acid supplementation on osmotic tolerance were measured in M9 in the presence of 0.65 M NaCl. The same procedure for growth kinetic analysis as described above was carried out using 5 ml cultures. Three biological replicates were used in each condition.

2.3.5 Metabolite analysis

Single colonies were inoculated in 10 ml M9 media in test tubes, incubate at 37°C aerobically until OD_{600} reached ~1.0. The cells were centrifuged at 2,800× g for 10 minutes, supernatants were removed and the pellets were resuspended in M9 at an OD_{600} of ~10.0. Then 500 µl samples were inoculated into 50 ml M9 or M9 supplemented with 0.6 M NaCl in 250 ml screw-capped flasks at an OD_{600} of ~0.1, and incubated at 37°C with shaking (275 rpm). Two technical replicates were made for each sample. When OD_{600} of samples reached exponential phase (OD_{600} ~0.7 - 1), the two technical replicates with a total volume of 100 ml were combined and cells were harvested by centrifugation at 9,000× g, 1 ml supernatant with extracellular metabolites was filtered using 0.2 µm syringe filter (VWR) and kept at -20°C. The remaining supernatants were removed completely. The cell pellets were washed with 5 ml fresh media, and then the supernatants were removed completely. Intracellular metabolites were extracted by suspending the cell pellets in 500 µl -20°C intracellular metabolites extraction buffer (acetonitrile: methanol: water (40: 40: 20, v: v: v)) (95) and incubated at -20°C for 30

minutes. Then samples were centrifuged (9,000× g) at 4°C for 5 minutes, and the supernatant was kept at -20°C for HPLC analysis. This extraction step was repeated for another two times using 400 µl and 300 µl intracellular metabolites extraction buffer (acetonitrile: methanol: water (40: 40: 20, v: v: v)) respectively with 15 minutes incubation at -20°C and then centrifuged (9,000× g) at 4°C for 5 minutes. The supernatants from the three extraction steps were combined. Then 500 µl water was combined with l ml extracted intracellular metabolites, filtered using 0.2 µm syringe filter (VWR) and kept at -20°C for HPLC analysis. Six biological replicates were used in each condition.

Table 2.2 Reagents used in analysis of amino acids.

Reagents	Composition
Mobile phase A	10 mM Na_2HPO_4: 10 mM $Na_2B_4O_7$: 5 mM NaN_3, pH 8.2
Mobile phase B	acetonitrile: methanol: water (45: 45: 10, v: v: v)
Borate buffers	0.4 M in water, pH 10.2
FMOC	2.5 mg ml^{-1} in acetonitrile
OPA	10 mg ml^{-1} Phthaldialdehuyde and 3-Mercaptopropionic acids in 0.4 M borate buffer

Trehalose, glucose, and acetic acid were analyzed using Aminex HPX-87H ion exclusion column (Bio-Rad, Hercules, CA) operated at 50°C with 5 mM sulfuric acid as the mobile phase at a flow rate of 0.6 ml min^{-1}, and detected by RI detector. Free amino acids were analyzed using modified Agilent amino acid analysis method (96). We used high-performance liquid chromatography (HPLC; Agilent Technologies, 1260 Infinity, Santa Clara, CA) and Cogent Bidentate C18™ (4 µm, 100 Å. Dimensions: 4.6 mm i.d. x

150 mm) column at 40°C. Flow rate of mobile phase was 0.6 ml min^{-1}. The mobile phase and reagents are listed in Table 2.2. Injection program and mobile phase gradients are listed in Table 2.3 and Table 2.4. The detection wavelength was 338 nm from 0 - 18 min, and 262 nm from 18 - 30 min.

Table 2.3 Injection program.

Steps	Injection program
1	Draw 12.5 µl from borate vial.
2	Draw 5 µl from sample vial.
3	Mix 17.5 µl from air 5 times.
4	Wait 0.2 min.
5	Draw 2.5 µl from OPA vial.
6	Mix 20 µl from air 10 times default speed.
7	Draw 2 µl from FMOC vial.
8	Mix 22 µl from air 10 times default speed.
9	Inject.
10	Wait 0.1 min.
11	Valve bypass.

Table 2.4 Mobile phase gradients.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	98	2
0.5	98	2
20	43	57
20.1	0	100
23.5	0	100
23.6	98	2
25	98	2
35	98	2

2.3.6 Effects of acetic acid supplementation

For experiments with acetic acid supplementation, 10 mM of acetic acid was used. The starting pH of the media with 10 mM acetic acid addition was ~6.5. Cells were cultivated as described in the metabolites analysis section above with 5 ml media in screw-capped tubes. Three biological replicates were used in each condition.

2.3.7 Cell membrane damage analysis

Cell membrane perturbation was analyzed by measuring the uptake of the fluorescent dye propidium iodide (PI). The OD_{600} of cells grown in M9 was adjusted to ~0.6, treated with M9 only, M9 supplemented with 0.7 M NaCl, M9 supplemented with 10 mM acetic acid, or M9 supplemented with 0.7 M NaCl and 10 mM acetic acid for 30 minutes at 37°C. The treated cells were centrifuged at 21,130× g for 2 minutes, and pellets were resuspended in PBS. PI staining was performed as previously described (97, 98). Briefly, PI was added to cells at a final concentration of 2.9 µM and incubated in the dark for 10 minutes at room temperature, then the cells were washed twice with PBS. To quantify uptake of PI, 200 µl of each sample was placed in black-walled 96-well plates (Greiner) and the fluorescence of PI staining was measured using a fluorescent plate reader (Molecular Devices SpectraMax® Gemini EM) using excitation wavelength of 495 nm and emission wavelength of 615 nm. The background fluorescence was corrected by subtracting the fluorescence of cells without PI straining. All fluorescence data were normalized by OD_{600}. Three biological replicates were used in each condition.

2.3.8 Transcriptional analysis

Perturbations to transcriptional regulation in the presence of 0.6 M NaCl were determined using microarray analysis using strains BW25113, EJW3, MG1655, and EYG1 with two biological replicates each. Samples were cultivated using the same condition as in metabolite analysis with 25 ml cultures and an initial OD_{600} of ~0.05. When OD_{600} reached ~0.5, cells were quickly chilled to ≤ 4°C on dry ice/isopropanol bath, then harvested by centrifugation (4,470× g) at 4°C followed by immediate resuspension in 5 ml of RNAlater (Sigma). Total RNA was extracted by using the ZR Fungal/Bacterial RNA MicroPrep™ (Zymo Research) kit. 10 μg of the isolated total RNA was mixed with 1.5 μg random primers (Promega), incubated at 70°C for 10 minutes and then cooled on ice (4°C). The cDNA was synthesized by combining the total RNA mixture with 10U SuperScript® III reverse transcriptase (Invitrogen), 1x first strand buffer (Invitrogen), 0.01 M DTT (Invitrogen), and nucleotides (0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dCTP, 0.2 mM dTTP [Promega] and 0.3 mM amino-allyl dUTP [Thermo Scientific]), and incubated at 42ºC for 3 hours. The cDNA was recovered with ice-cold ethanol precipitation, labeled with either Cy3- or Cy5- mono-reactive dye (GE Healthcare), and then hybridized to the *E. coli* Gene Expression Microarray (Agilent Technologies). The arrays were scanned using the GenePix 4100A Microarray Scanner and the images were analyzed using GenePix Pro 6.0 software (Molecular Devices). The MIDAS software package (TM4) (99) was used to normalize the data using LOWESS based normalization algorithm (100). The rank product method with a critical *p*-value < 0.01 was used to identify the differentially expressed genes using MeV (TM4)

microarray analysis software (99). Gene ontology analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (101, 102).

2.3.9 Overexpression and deletion assay

Genes uniquely upregulated or downregulated in the *rpoC* mutant in the BW25113 background were further investigated via overexpression or deletion studies. Plasmids from the ASKA collection (103) were transformed into BW25113 for overexpression assays. The growth kinetics of the overexpression strains were compared against the wild-type strain expressing the empty vector pCA24N in M9 with or without 0.55 M NaCl supplementation. Knockout strains were obtained from the Keio collection (93). The kanamycin resistance marker in the Keio strains was removed by transforming with the plasmid pCP20 as previously described (104), and the marker-less knockout strains were used for deletion assay using BW25113 as the negative control in M9 with or without 0.6 M NaCl supplementation. Three biological replicates were used in each condition.

2.3.10 Microarray data accession number

Microarray data have been deposited in the Gene Expression Omnibus (GEO) database with accession number GSE94342.

2.4 Result and discussion

2.4.1 The rpoC K370_A396dup mutation confers osmotic tolerance in BW25113

Our prior work investigating osmotic tolerance in *E. coli* using adaptive laboratory evolution yielded several mutants with significantly increased tolerance to NaCl stress (79). Two isolated osmotolerant mutants (G3 and G4) share an identical

rpoC K370_A396dup mutation. These mutants were isolated from independent populations, which led us to hypothesize that this specific mutation is a causative mutation for the improved osmotic tolerance observed. Since both G3 and G4 also contain additional mutations, in order to study the specific function of the *rpoC* K370_A396dup mutation, we reconstructed this mutation in the BW25113 wild-type background to generate strain EJW3. In addition, since the parental strain from which G3 and G4 mutants were derived contains a *trpB* deletion, we also generated strain EJW4 by deleting the *trpB* gene from EJW3.

The growth kinetics of strain BW25113, EJW3 (BW25113 *rpoC* K370_A396dup), JW1253 (BW25113 Δ*trpB769::kan*), EJW4 (BW25113 *rpoC* K370_A396dup, Δ*trpB769::kan*), G3 (evolved osmotolerant mutant containing *rpoC* K370_A396dup mutation, BW25113 background), and Hfr-2×SFX- (ancestor of G3, BW25113 background) (79) were compared in M9 supplemented with 0.6 M NaCl and 50 μg ml^{-1} tryptophan (Figure 2.1). Since BW25113 and EJW3 are prototrophic for tryptophan, we also compared the growth of these two strains in the absence of tryptophan to assess the impact of the *rpoC* K370_A396dup mutation on osmotic tolerance in the absence of amino acid supplementation. The results showed that in the absence of tryptophan supplementation, the growth of BW25113 was drastically inhibited by 0.6 M NaCl 4 hours after incubation with no significant growth observed within the next 20 hours, while strain EJW3 continued to grow and reached significantly higher biomass than BW25113 after 24 hours. The data showed that the addition of tryptophan significantly increased the growth of both BW25113 and the reconstructed

rpoC mutant EJW3 in the presence of 0.6 M NaCl (Figure 2.1A). Though the addition of tryptophan improved the performance of all strains tested in the presence of 0.6 M NaCl, strains with the *rpoC* K370_A396dup mutation (EJW3, EJW4, and G3) still outperformed their *rpoC* wild-type counterparts (Figure 2.1), strongly suggesting that the *rpoC* K370_A396dup mutation is a causative mutation for enhanced osmotic tolerance. Interestingly, the level of osmotic tolerance conferred by the *rpoC* mutation alone was on the same level as that conferred by the addition of 50 µg ml^{-1} tryptophan. Results in Figure 2.1 showed no significant difference in specific growth rates during the first few hours of growth between BW25113 and EJW3, therefore biomass concentrations after 24 and 48 hours were used to assess osmotic tolerance for the majority of this work.

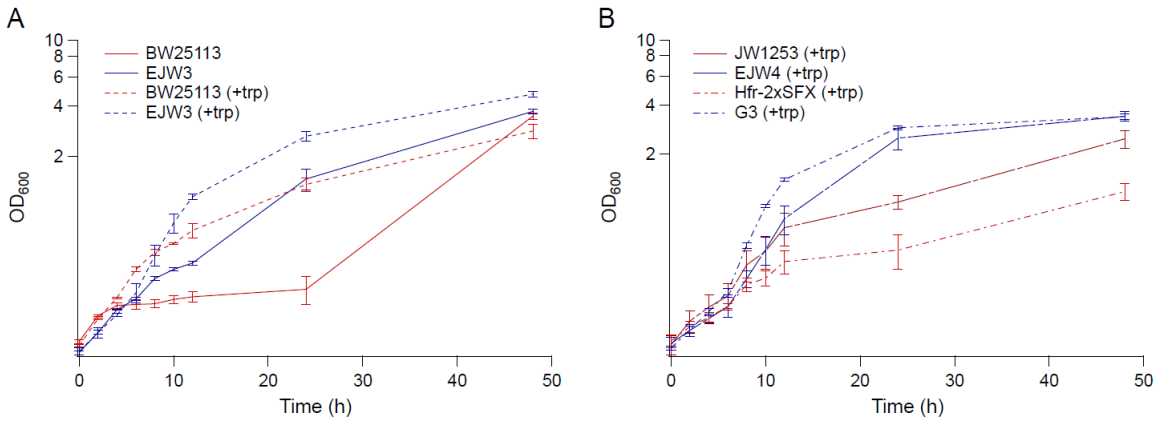

Figure 2.1. Growth kinetics in M9 supplemented with 0.6 M NaCl. (A) BW25113 and EJW3 in M9 supplemented with 0.6 M NaCl [solid lines: without tryptophan, and dashed lines: with 50 µg ml^{-1} tryptophan supplementation]. (B) Tryptophan auxotrophic strains JW1253, EJW4, HFr-2×SFX- and G3 in M9 supplemented with 0.6 M NaCl and 50 µg ml^{-1} tryptophan. Red lines: strains without *rpoC* mutation. Blue lines: strains with *rpoC* mutation. Error bars are standard deviations.

To determine the impact of the *rpoC* mutation on cell viability in hyperosmotic stress, BW25113 and EJW3 were grown in M9 supplemented with 0.6 M NaCl and the concentration of viable cells were quantified over time (Figure 2.2). The concentration of viable cells of both strains decreased within the first few hours, indicating the occurrence of cell death upon initial exposure to hyperosmotic stress. However, increases in the concentration of viable cells were observed in EJW3 sooner and at a faster rate than BW25113. The same trend was also observed using OD_{600}, indicating that OD_{600} and the concentration of viable cells are correlated. The increase in growth (based on OD_{600} measurements) observed in hyperosmotic conditions when tryptophan was supplemented also correlated with increased cell viability. Furthermore, there was no observable differences in cell size or shape between BW25113 and EJW3 in the presence or absence of 0.6 M NaCl at different growth phases (Figure 2.3). Thus, OD_{600} was used as the measure of cell growth for the remainder of this study.

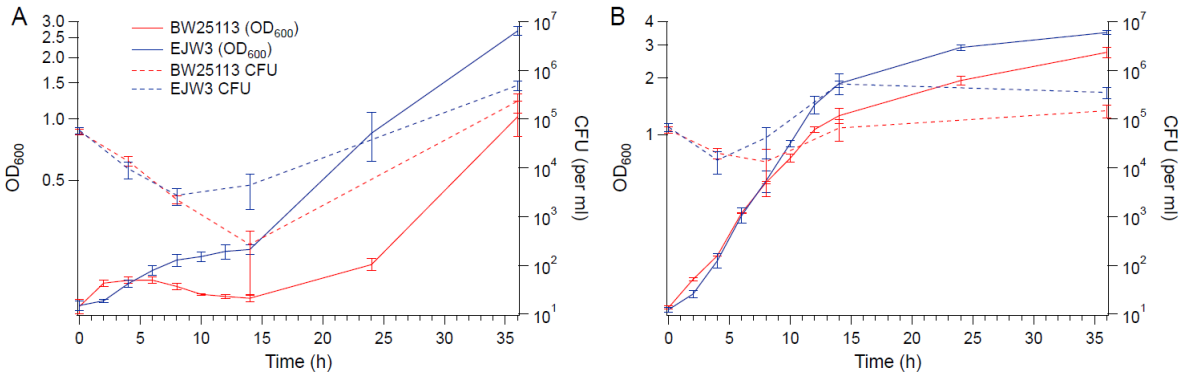

Figure 2.2. Comparison between OD_{600} and viability. (A) M9 supplemented with 0.6 M NaCl. (B) M9 supplemented with 0.6 M NaCl and 50 µg tryptophan ml^{-1}.

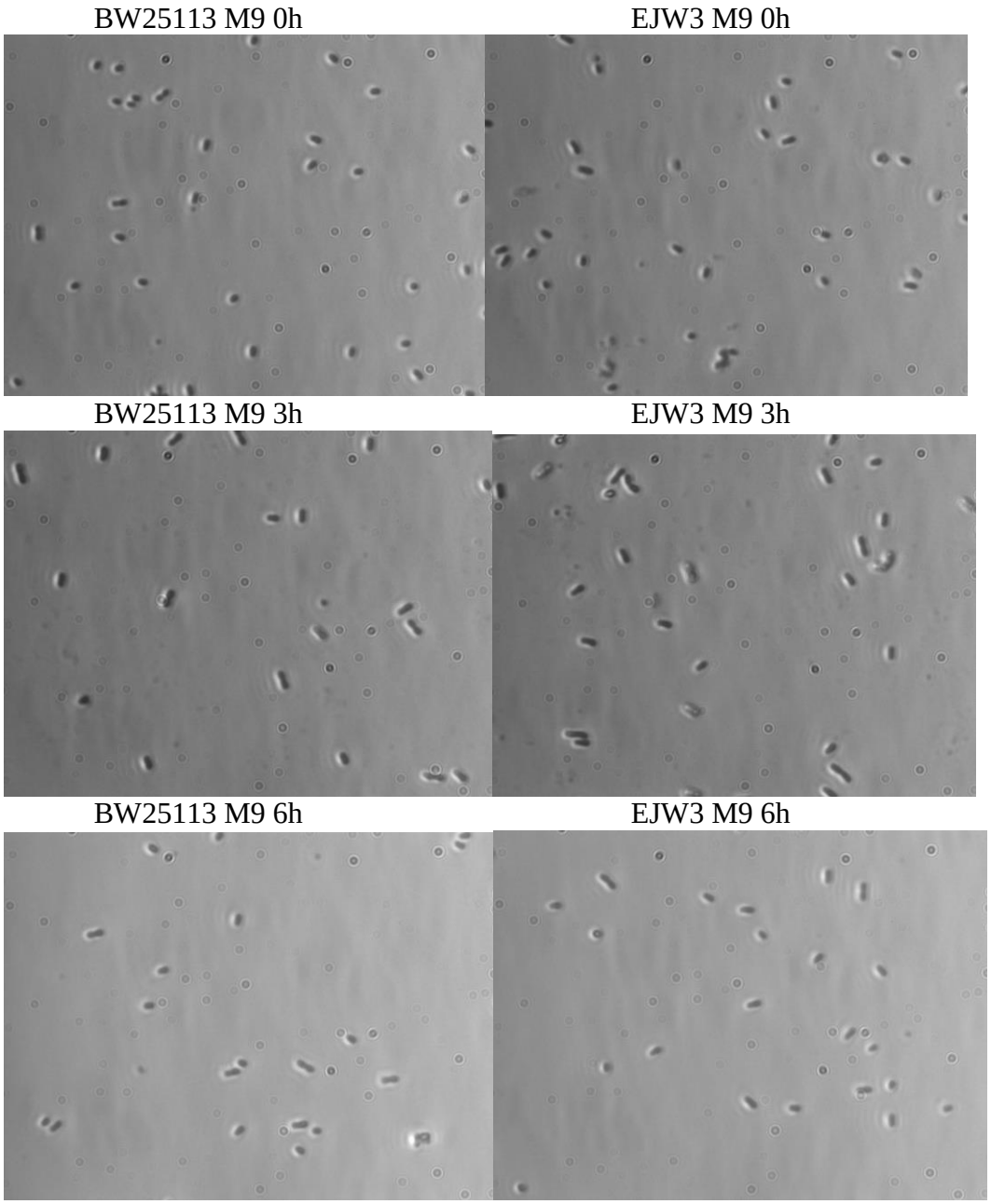

Figure 2.3. Light microscopy of cells in the presence or absence of hyperosmotic stress.

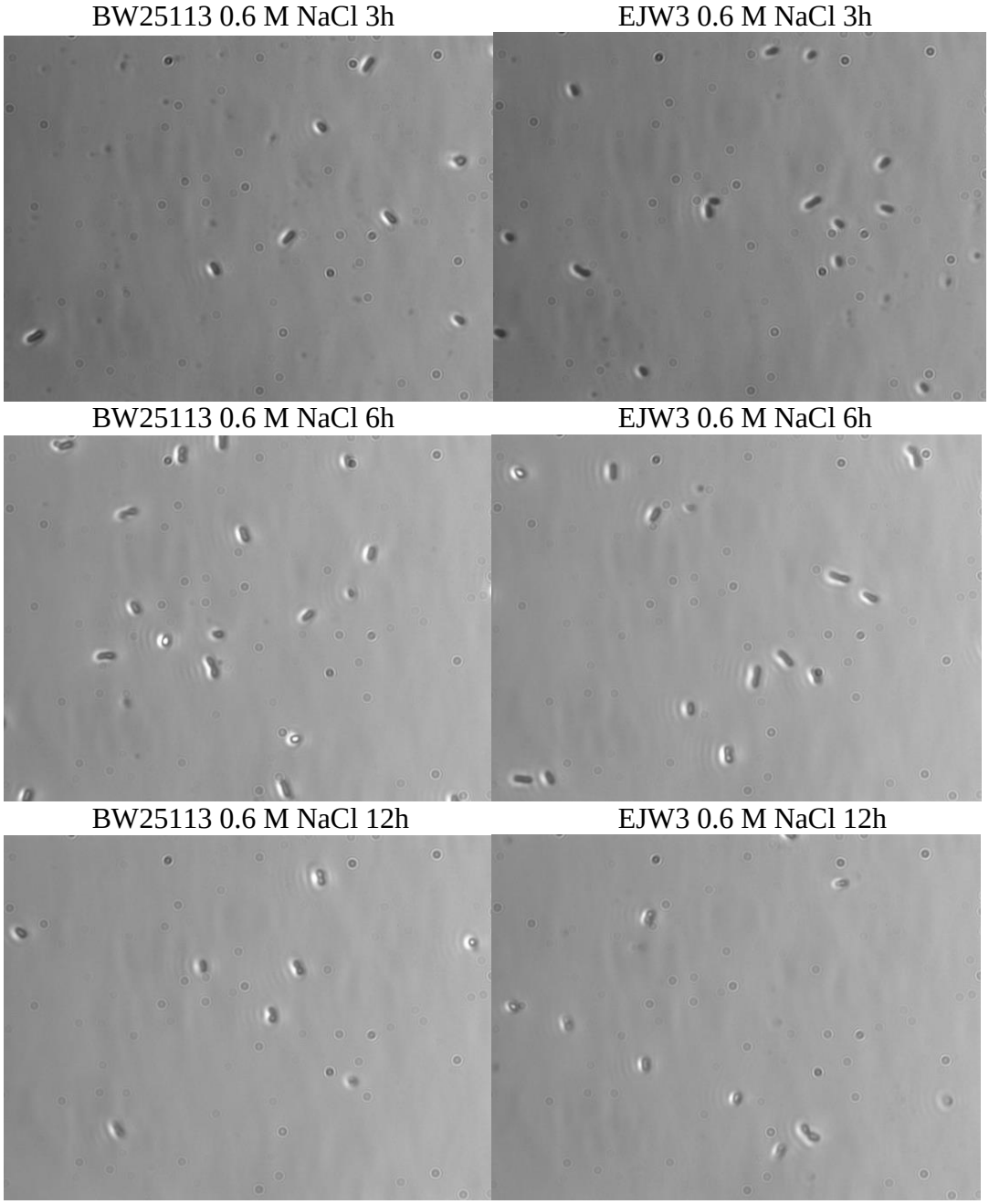

Figure 2.3. Continued.

BW25113 0.6 M NaCl 24h EJW3 0.6 M NaCl 24h

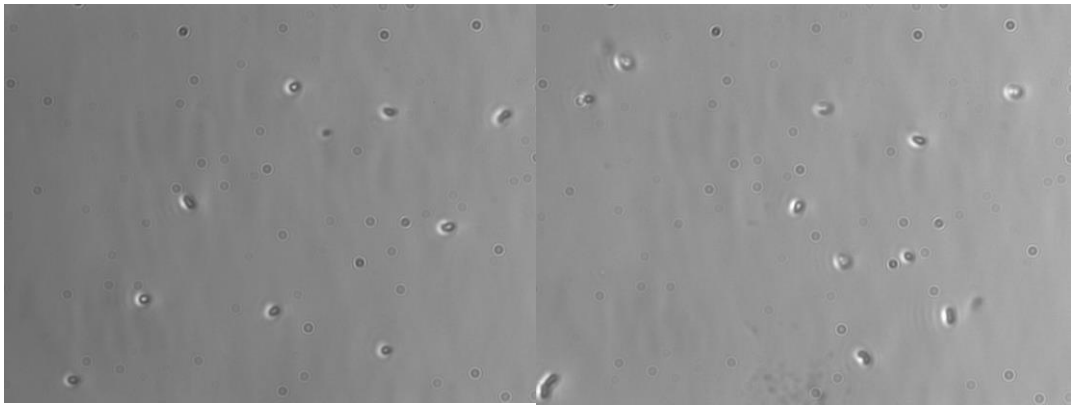

Figure 2.3. Continued.

The impact of the *rpoC* mutation on tolerance to higher osmotic stress was tested in both aerobic and micro-aerobic conditions. Preliminary tests showed higher osmotic tolerance of the strains in micro-aerobic vs. aerobic conditions, therefore 0.8 M, 0.9 M, and 1 M NaCl were used in micro-aerobic conditions, while 0.75 M, 0.8 M, and 0.9 M NaCl were used under aerobic conditions. The higher osmotic tolerance observed under micro-aerobic conditions is likely due to higher induction of the osmotic tolerance related genes *ompC* and *proU* under anaerobic conditions (105). Relative growth results in 0.8 M (micro-aerobic) and 0.75 M (aerobic) NaCl are shown in Tables 2.5 and 2.6. In micro-aerobic and aerobic conditions, all strains with the *rpoC* mutation showed significant growth in the presence of 0.8 M and 0.75 M NaCl respectively, while only slight growth was observed in their wild-type counterparts. In the presence of 0.9 M NaCl in micro-aerobic condition and 0.8 M NaCl in aerobic condition, only slight

turbidity was observed in all strains (Tables 2.7 and 2.8), and no growth was observed in any strain cultured micro-aerobically in the presence of 1 M NaCl or aerobically with 0.9 M NaCl.

Table 2.5 Growth in micro-aerobic condition in M9 supplemented with 0.8 M NaCl.

Strains	0 h	24 h	48 h	72 h
BW25113	-	+	+	+
EJW3	-	+	+++	++++
JW1253*	-	+	++	+
EJW4*	-	++	++++	++++
Hfr-2×SFX-*	-	+	+	+
G3*	-	+++	++++	+++

* Tryptophan (50 μg ml^{-1}) was supplemented

- OD600 < 0.1, + 0.1 < OD600 < 0.5, ++ 0.5 < OD600 < 1.0, +++ 1.0 < OD600 < 2.0, ++++ OD600 > 2.0

Table 2.6 Growth in aerobic condition in M9 supplemented with 0.75 M NaCl.

Strains	0 h	24 h	48 h	72 h
BW25113	-	+	-	-
EJW3	-	+	++++	++++
JW1253*	-	+	++	++
EJW4*	-	++	++++	++++
Hfr-2×SFX-*	-	+	+	+
G3*	-	+++	++++	++++

* Tryptophan (50 μg ml^{-1}) was supplemented

- OD600 < 0.1, + 0.1 < OD600 < 0.5, ++ 0.5 < OD600 < 1.0, +++ 1.0 < OD600 < 2.0, ++++ OD600 > 2.0

Table 2.7 Growth in micro-aerobic condition in M9 supplemented with 0.9 M NaCl.

Strains	0 h	24 h	48 h	72 h
BW25113	-	+	-	-
EJW3	-	+	-	-
JW1253*	-	+	+	+
EJW4*	-	+	+	+
Hfr-2×SFX-*	-	+	+	+
G3*	-	+	+	+

* Tryptophan (50 µg ml^{-1}) was supplemented

- OD600 < 0.1, + 0.1 < OD600 < 0.5, ++ 0.5 < OD600 < 1.0, +++ 1.0 < OD600 < 2.0, ++++ OD600 > 2.0

Table 2.8 Growth in aerobic condition in M9 supplemented with 0.8 M NaCl.

Strains	0 h	24 h	48 h	72 h
BW25113	-	-	-	-
EJW3	-	+	-	-
JW1253*	-	+	+	+
EJW4*	-	+	+	+
Hfr-2×SFX-*	-	+	-	-
G3*	-	+	+	+

* Tryptophan (50 µg ml^{-1}) was supplemented

- OD600 < 0.1, + 0.1 < OD600 < 0.5, ++ 0.5 < OD600 < 1.0, +++ 1.0 < OD600 < 2.0, ++++ OD600 > 2.0

2.4.2 Effects of amino acid supplementation

Since we observed an improvement in growth performance under osmotic stress with tryptophan supplementation, and prior work have reported the impact of some amino acids (*e.g.* glutamic acid, proline) on osmotic stress response (86, 106), we hypothesized that there are additional amino acids that also play a role in osmotic tolerance in *E. coli*. Thus, we assessed the impact of individual amino acid

Table 2.9 Cell density (OD_{600}) after 24 and 48 hours of growth in M9 supplemented with 0.65 M NaCl and amino acids supplemented at specified concentrations.

Amino acid	Concentration (μg ml^{-1})	24h		48h	
		BW25113	EJW3	BW25113	EJW3
Control[a]	0	0.22 ± 0.09	0.70 ± 0.31	0.90 ± 0.45	3.03 ± 0.30
Ala	10	0.34 ± 0.09	1.19 ± 0.44	0.80 ± 0.44	2.92 ± 0.29
	100	**1.77 ± 0.58***	**2.78 ± 0.30***	**2.20 ± 0.10***	3.11 ± 0.15
	1000	0.93 ± 0.33	***0.22 ± 0.10****	**4.13 ± 0.47***	***0.45 ± 0.26****
Arg	10	***0.14 ± 0.01****	1.76 ± 0.53	0.71 ± 0.16	3.57 ± 0.38
	100	**1.70 ± 0.44***	**2.84 ± 0.17***	**2.90 ± 0.33***	**3.77 ± 0.20***
	1000	**2.40 ± 0.26***	**2.69 ± 0.26***	**2.93 ± 0.58***	3.23 ± 0.29
Asn	10	**0.41 ± 0.02***	**1.90 ± 0.42***	**2.63 ± 0.14***	3.49 ± 0.25
	100	**3.16 ± 0.30***	**3.04 ± 0.19***	**3.30 ± 0.10***	**4.02 ± 0.15***
	1000	**3.04 ± 0.46***	**3.45 ± 0.29***	**3.44 ± 0.22***	4.12 ± 0.52
Asp	10	0.33 ± 0.08	0.60 ± 0.12	1.02 ± 0.46	***2.77 ± 0.10****
	100	**1.82 ± 0.29***	**2.77 ± 0.21***	**2.50 ± 0.09***	3.08 ± 0.32
	1000	**2.21 ± 0.52***	**3.13 ± 0.09***	**2.27 ± 0.31***	**3.65 ± 0.22***
Cys	10	**2.68 ± 0.25***	**2.95 ± 0.25***	**3.10 ± 0.24***	**4.04 ± 0.25***
	100	**0.78 ± 0.22***	**1.60 ± 0.12***	**2.68 ± 0.05***	***2.33 ± 0.04****
	1000	***0.10 ± 0.01****	***0.09 ± 0.01****	1.90 ± 0.78	1.05 ± 1.47
Glu	10	0.19 ± 0.03	**1.16 ± 0.10***	**1.46 ± 0.12***	2.84 ± 0.19
	100	**1.88 ± 0.44***	**2.64 ± 0.11***	**2.56 ± 0.18***	3.04 ± 0.19
	1000	**2.48 ± 0.39***	**2.74 ± 0.12***	**3.33 ± 0.51***	3.35 ± 0.31
Gln	10	0.29 ± 0.08	0.77 ± 0.16	0.98 ± 0.38	3.01 ± 0.29
	100	**0.94 ± 0.10***	**2.76 ± 0.06***	**2.69 ± 0.13***	**3.30 ± 0.14***
	1000	**2.75 ± 0.41***	**3.19 ± 0.52***	**3.09 ± 0.22***	**3.86 ± 0.35***
Gly	10	**0.40 ± 0.01***	**1.88 ± 0.05***	**1.85 ± 0.14***	3.37 ± 0.19
	100	**2.01 ± 0.14***	**3.17 ± 0.23***	**2.79 ± 0.07***	**3.83 ± 0.05***
	1000	0.50 ± 0.13	**2.17 ± 0.36***	***0.28 ± 0.07****	**3.52 ± 0.09***
His	10	**2.38 ± 0.31***	**3.05 ± 0.20***	**3.27 ± 0.18***	**4.15 ± 0.18***
	100	**2.48 ± 0.23***	**2.80 ± 0.15***	**3.24 ± 0.07***	**3.85 ± 0.26***
	1000	**2.63 ± 0.21***	**2.96 ± 0.10***	**3.12 ± 0.30***	**3.54 ± 0.12***
Ile	10	0.23 ± 0.04	**1.72 ± 0.14***	**1.29 ± 0.13***	3.15 ± 0.23
	100	**2.04 ± 0.07***	**2.97 ± 0.07***	**1.81 ± 0.16***	**3.69 ± 0.16***
	1000	**0.39 ± 0.05***	***0.21 ± 0.02****	***0.46 ± 0.06****	***0.22 ± 0.02****
Leu	10	0.62 ± 0.19	**1.51 ± 0.06***	**2.28 ± 0.53***	3.15 ± 0.04
	100	**1.97 ± 0.14***	**1.78 ± 0.21***	**2.52 ± 0.14***	***2.04 ± 0.10****
	1000	**0.40 ± 0.08***	***0.27 ± 0.04****	***0.36 ± 0.11****	***0.27 ± 0.05****

Table 2.9 Continued

Amino acid	Concentration (μg ml^{-1})	24h		48h	
		BW25113	EJW3	BW25113	EJW3
Lys	10	0.27 ± 0.03	0.77 ± 0.10	1.00 ± 0.82	2.92 ± 0.07
	100	**1.41 ± 0.27***	**2.75 ± 0.24***	**2.47 ± 0.15***	3.11 ± 0.21
	1000	**2.79 ± 0.15***	**2.34 ± 0.06***	**2.66 ± 0.37***	3.12 ± 0.50
Met	10	0.23 ± 0.02	0.57 ± 0.06	***0.18 ± 0.01****	2.68 ± 0.20
	100	**0.31 ± 0.01***	**1.87 ± 0.31***	***0.25 ± 0.10****	***1.84 ± 0.07****
	1000	0.18 ± 0.00	***0.27 ± 0.10****	***0.09 ± 0.01****	***0.21 ± 0.11****
Phe	10	**0.36 ± 0.02***	**2.19 ± 0.16***	**2.25 ± 0.36***	3.32 ± 0.33
	100	**2.69 ± 0.20***	**2.84 ± 0.06***	**3.37 ± 0.37***	**3.62 ± 0.04***
	1000	**2.88 ± 0.27***	**2.66 ± 0.08***	**3.83 ± 0.07***	**4.08 ± 0.04***
Pro	10	**0.39 ± 0.01***	**1.00 ± 0.09***	0.89 ± 0.12	3.26 ± 0.24
	100	**2.27 ± 0.80***	***0.23 ± 0.02****	**3.53 ± 0.10***	***0.42 ± 0.14****
	1000	1.25 ± 0.65	***0.21 ± 0.01****	**3.69 ± 0.12***	***0.58 ± 0.16****
Ser	10	***0.15 ± 0.01****	**1.60 ± 0.35***	0.79 ± 0.03	3.21 ± 0.24
	100	**2.70 ± 0.33***	**2.96 ± 0.28***	**3.29 ± 0.20***	**3.91 ± 0.06***
	1000	0.27 ± 0.02	***0.45 ± 0.08****	***0.27 ± 0.02****	***0.52 ± 0.11****
Thr	10	**0.44 ± 0.07***	1.28 ± 0.31	**2.20 ± 0.24***	3.08 ± 0.12
	100	**2.80 ± 0.54***	**2.92 ± 0.01***	**2.59 ± 0.34***	**3.43 ± 0.08***
	1000	0.28 ± 0.11	***0.16 ± 0.02****	0.51 ± 0.51	***0.31 ± 0.15****
Trp	10	**0.52 ± 0.06***	**2.48 ± 0.26***	**2.31 ± 0.12***	3.73 ± 0.41
	100	**2.60 ± 0.09***	**3.10 ± 0.14***	**3.36 ± 0.14***	**3.71 ± 0.12***
	1000	**1.85 ± 0.23***	**2.72 ± 0.09***	**2.76 ± 0.18***	**3.61 ± 0.27***
Tyr	10	**0.73 ± 0.12***	**2.67 ± 0.22***	**2.79 ± 0.16***	**3.76 ± 0.05***
	100	**2.78 ± 0.33***	**2.73 ± 0.35***	**3.74 ± 0.12***	3.29 ± 0.35
	400	**2.05 ± 0.41***	**1.94 ± 0.03***	**3.32 ± 0.06***	3.10 ± 0.11
Val	10	0.21 ± 0.01	***0.19 ± 0.00****	***0.20 ± 0.01****	***0.18 ± 0.06****
	20	0.23 ± 0.01	***0.19 ± 0.00****	***0.23 ± 0.01****	***0.19 ± 0.01****
	40	0.24 ± 0.01	***0.20 ± 0.01****	***0.25 ± 0.02****	***0.20 ± 0.02****

[a] Control is the condition with no addition of amino acid, and includes fifteen biological replicates from five batches, three biological replicates per batch.

* Statistically significantly different from control (p-value < 0.05). P-values are calculated using a two-tailed student's t-test. Significantly increased values are bolded. Significantly reduced values are bolded and italicized.

supplementation on osmotic tolerance of BW25113 and EJW3. Preliminary data with tryptophan supplementation showed a more observable benefit in the presence of higher NaCl concentration, therefore a higher osmotic stress with 0.65 M NaCl was used. The summary of amino acid supplementation on optical cell density (OD_{600}) is listed in Table 2.9. With a few exceptions (valine, methionine, proline, alanine), the addition of 10 μg ml^{-1} or 100 μg ml^{-1} of most amino acids tested significantly improved the growth of both BW25113 and EJW3 under osmotic stress. This may be partially due to the decreased ATP requirements for amino acid biosynthesis when these amino acids are supplemented (107), which also is the likely reason that higher osmotic tolerance is observed when *E. coli* is grown on rich media than in minimal media without amino acids. However, higher concentrations of some amino acids (*e.g.* serine, cysteine, threonine, isoleucine, and leucine) reduced growth, likely due to feedback inhibition. Interestingly, the addition of 100 μg ml^{-1} methionine appears to result in a faster accumulation of biomass (based on the higher biomass concentration at 24 hours), but a 39% lower final biomass (p-value < 0.001) after 48 hours in strain EJW3. However, the addition of 100 μg ml^{-1} methionine led to a 72% lower final biomass (p-value < 0.001) in BW25113 after 48 hours, which suggests a higher feedback inhibition of methionine in strain BW25113 compared with EJW3 (Figure 2.4). The supplementation of methionine has been reported to improve the tolerance to NaCl in *Saccharomyces cerevisiae* (108), but has not been reported in *E. coli*. Our results suggest potential synergy between the *rpoC* mutation and methionine supplementation on osmotic tolerance in *E. coli*. The supplementation with 100 μg ml^{-1} and 1000 μg ml^{-1} of proline (Figure 2.5) or with 1000

µg ml^{-1} of alanine (Figure 2.6) resulted in significant growth inhibition in EJW3, but were beneficial to BW25113, in the presence of osmotic stress. This led us to hypothesize that EJW3 may produce more proline and alanine, making it more sensitive to additional supplementation of these two amino acids, compared with BW25113. Proline has been reported as an osmoprotectant in *E. coli* (106, 109) and other microorganisms (110). Thus, the overproduction of proline may be one of the causes that contribute to the higher osmotic tolerance in EJW3.

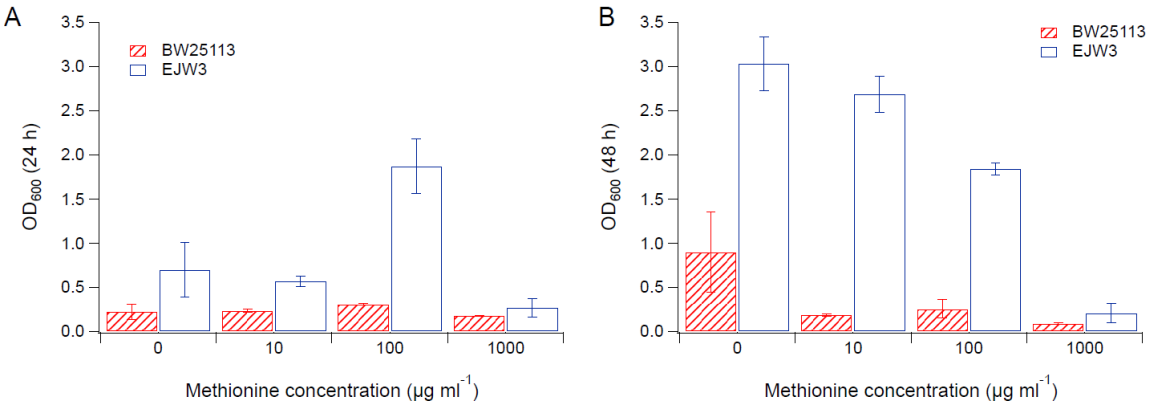

Figure 2.4. Effects of methionine supplementation on growth in the presence of 0.65 M NaCl. (A) Optical cell density (OD_{600}) after 24 hours. (B) Optical cell density (OD_{600}) after 48 hours. Error bars are standard deviations.

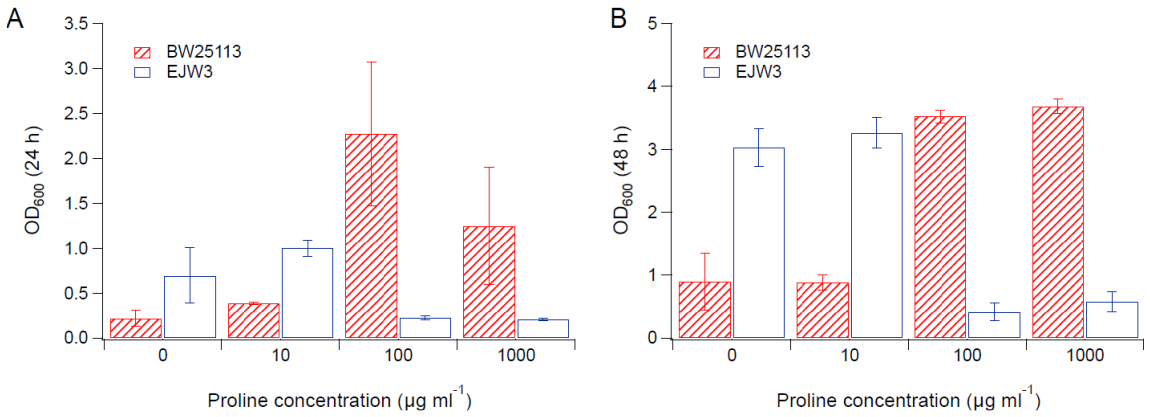

Figure 2.5. Effects of proline supplementation on growth in the presence of 0.65 M NaCl. (A) Optical cell density (OD_{600}) after 24 hours. (B) Optical cell density (OD_{600}) after 48 hours. Error bars are standard deviations.

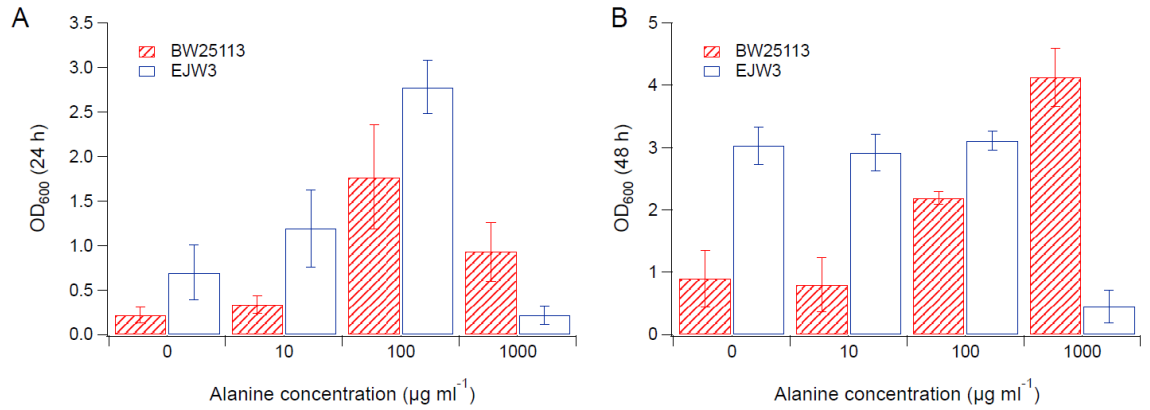

Figure 2.6. Effects of alanine supplementation on growth in the presence of 0.65 M NaCl. (A) Optical cell density (OD_{600}) after 24 hours. (B) Optical cell density (OD_{600}) after 48 hours. Error bars are standard deviations.

2.4.3 Metabolite analysis

In order to test our hypothesis that *rpoC* K370_A396dup mutation led to an overproduction of some amino acids such as proline and alanine, and to identify other potential effects of the mutation on the production of other metabolites, extracellular and

Table 2.10 Quantification of intracellular and extracellular metabolites in M9 only.

	Metabolite	BW25113	EJW3	*P*-value
Intracellular	Trehalose (µg ml $^{-1}$ OD_{600}^{-1})	-	-	-
	Glucose (µg ml $^{-1}$ OD_{600}^{-1})	770 ± 140	800 ± 110	0.706
	Acetic acid (µg ml $^{-1}$ OD_{600}^{-1})	650 ± 90	***390 ± 80***	0.000*
	Asp (µg ml^{-1} OD_{600}^{-1})	-	-	-
	Glu (µg ml^{-1} OD_{600}^{-1})	187.72 ± 35.94	212.37 ± 27.2	0.210
	Asn Ser (µg ml^{-1} OD_{600}^{-1})	9.45 ± 0.75	10.11 ± 0.94	0.204
	Gln Gly His Thr (µg ml^{-1} OD_{600}^{-1})	10.82 ± 0.67	10.14 ± 2.49	0.534
	Ala (µg ml^{-1} OD_{600}^{-1})	81.18 ± 16.55	83.71 ± 3.15	0.721
	Arg (µg ml^{-1} OD_{600}^{-1})	-	4.78 ± 7.41	0.145
	Tyr (µg ml^{-1} OD_{600}^{-1})	-	4.10 ± 10.04	0.341
	Val (µg ml^{-1} OD_{600}^{-1})	-	-	-
	Met (µg ml^{-1} OD_{600}^{-1})	55.11 ± 6.01	**74.52 ± 10.88**	0.003*
	Trp (µg ml^{-1} OD_{600}^{-1})	-	-	-
	Phe Ile (µg ml^{-1} OD_{600}^{-1})	-	-	-
	Leu (µg ml^{-1} OD_{600}^{-1})	3.83 ± 9.38	17.56 ± 13.81	0.072
	Lys (µg ml^{-1} OD_{600}^{-1})	6.95 ± 10.78	7.08 ± 11.24	0.983
	Pro (µg ml^{-1} OD_{600}^{-1})	61.64 ± 6.12	**75.06 ± 1.95**	0.000*
Extracellular	Trehalose (µg ml $^{-1}$ OD_{600}^{-1})	-	-	-
	Glucose (µg ml $^{-1}$ OD_{600}^{-1})	5280 ± 300	5380 ± 140	0.466
	Acetic acid (µg ml $^{-1}$ OD_{600}^{-1})	280 ± 20	**370 ± 10**	0.000*
	Asp (µg $ml^{-1}OD_{600}^{-1}$)	-	-	-
	Glu (µg $ml^{-1}OD_{600}^{-1}$)	-	6.42 ± 12.41	0.234
	Asn Ser (µg ml^{-1} OD_{600}^{-1})	-	-	-
	Gln Gly His Thr (µg ml^{-1} OD_{600}^{-1})	-	-	-
	Ala (µg ml^{-1} OD_{600}^{-1})	10.86 ± 6.33	***1.93 ± 4.72***	0.020*
	Arg (µg ml^{-1} OD_{600}^{-1})	2.95 ± 7.21	-	0.341
	Tyr (µg ml^{-1} OD_{600}^{-1})	26.01 ± 14.8	**45.14 ± 5.72**	0.014*
	Val (µg ml^{-1} OD_{600}^{-1})	-	-	-

Table 2.10 Continued

	Metabolite	BW25113	EJW3	*P*-value
Extracellular	Met (μg ml^{-1} OD_{600}^{-1})	3.92 ± 9.60	**41.35 ± 11.43**	0.000*
	Trp (μg ml^{-1} OD_{600}^{-1})	24.85 ± 60.86	**131.60 ± 90.56**	0.038*
	Phe Ile (μg ml^{-1} OD_{600}^{-1})	89.07 ± 45.53	***20.37 ± 49.90***	0.032*
	Leu (μg ml^{-1} OD_{600}^{-1})	-	-	-
	Lys (μg ml^{-1} OD_{600}^{-1})	-	-	-
	Pro (μg ml^{-1} OD_{600}^{-1})	516.13 ± 35.09	**776.02 ± 117.02**	0.000*

- Not detectible.
* Statistically significantly different between BW25113 and EJW3 (*p*-values < 0.05). Values bolded are significantly higher in EJW3. Values bolded and italicized are significantly lower in EJW3. *P*-values are calculated by using a two-tailed student's *t*-test with six biological replicates.

intracellular metabolites of BW25113 and EJW3 during late exponential phase (OD_{600} about 0.7 - 1.0) in the presence and absence of osmotic stress were analyzed. The results are shown in Tables 2.10 and 2.11. The results showed that strain EJW3 produced ~22% more intracellular (*p*-value < 0.001) and ~50% more extracellular (*p*-value < 0.001) proline than BW25113 in the absence of hyperosmotic stress, while the exposure to 0.6 M NaCl led to increased intracellular amount of proline in both strains and extracellular amount of proline produced by BW25113. Prior study has shown the overproduction of proline to confer enhanced osmotic stress tolerance (111). Since the inoculum used in our study were prepared without the addition of excess NaCl, the EJW3 culture likely contained higher initial intracellular proline than BW25113, which may result in early protection to EJW3 from the inhibition of 0.6 M NaCl. Glutamic acid has been found to accumulate in osmotically stressed cells and serves as an osmoprotectant (86). Consistent with prior work, results from our experiment also showed an increase in

Table 2.11 Quantification of intracellular and extracellular metabolites in M9 supplemented with 0.6 M NaCl.

	Metabolite	BW25113	EJW3	*P*-value
Intracellular	Trehalose (μg ml $^{-1}$ OD_{600}^{-1})	1190 ± 190	**1630 ± 220**	0.005*
	Glucose (μg ml $^{-1}$ OD_{600}^{-1})	1050 ± 80	1030 ± 180	0.797
	Acetic acid (μg ml $^{-1}$ OD_{600}^{-1})	270 ± 140	370 ± 50	0.137
	Asp (μg ml^{-1} OD_{600}^{-1})	-	-	-
	Glu (μg ml^{-1} OD_{600}^{-1})	565.50 ± 44.39	**698.75 ± 82.47**	0.006*
	Asn Ser (μg ml^{-1} OD_{600}^{-1})	1.02 ± 2.50	**6.74 ± 3.71**	0.011*
	Gln Gly His Thr (μg ml^{-1} OD_{600}^{-1})	8.64 ± 1.59	9.69 ± 4.83	0.623
	Ala (μg ml^{-1} OD_{600}^{-1})	38.49 ± 9.03	**71.58 ± 13.51**	0.001*
	Arg (μg ml^{-1} OD_{600}^{-1})	28.79 ± 2.73	**44.87 ± 6.42**	0.000*
	Tyr (μg ml^{-1} OD_{600}^{-1})	-	-	-
	Val (μg ml^{-1} OD_{600}^{-1})	-	0.44 ± 1.08	0.341
	Met (μg ml^{-1} OD_{600}^{-1})	21.29 ± 6.35	**30.55 ± 2.53**	0.008*
	Trp (μg ml^{-1} OD_{600}^{-1})	-	-	-
	Phe Ile (μg ml^{-1} OD_{600}^{-1})	-	3.24 ± 5.27	0.164
	Leu (μg ml^{-1} OD_{600}^{-1})	-	-	-
	Lys (μg ml^{-1} OD_{600}^{-1})	-	9.08 ± 10.05	0.051
	Pro (μg ml^{-1} OD_{600}^{-1})	94.31 ± 6.14	107.31 ± 18.37	0.131
Extracellular	Trehalose (μg ml $^{-1}$ OD_{600}^{-1})	-	-	-
	Glucose (μg ml $^{-1}$ OD_{600}^{-1})	4950 ± 770	4600 ± 1000	0.518
	Acetic acid (μg ml $^{-1}$ OD_{600}^{-1})	260 ± 20	**360 ± 20**	0.000*
	Asp (μg ml^{-1} OD_{600}^{-1})	-	-	-
	Glu (μg ml^{-1} OD_{600}^{-1})	234.82 ± 15.44	***88.05 ± 19.38***	0.000*
	Asn Ser (μg ml^{-1} OD_{600}^{-1})	-	-	-
	Gln Gly His Thr (μg ml^{-1} OD_{600}^{-1})	-	-	-
	Ala (μg ml^{-1} OD_{600}^{-1})	-	-	-
	Arg (μg ml^{-1} OD_{600}^{-1})	-	7.90 ± 12.34	0.148
	Tyr (μg ml^{-1} OD_{600}^{-1})	41.73 ± 6.31	***16.05 ± 24.95***	0.035*

Table 2.11 Continued

	Metabolite	BW25113	EJW3	*P*-value
Extracellular	Val (μg ml^{-1} OD_{600}^{-1})	-	-	-
	Met (μg ml^{-1} OD_{600}^{-1})	40.03 ± 8.39	38.80 ± 18.26	0.884
	Trp (μg ml^{-1} OD_{600}^{-1})	88.49 ± 30.23	***20.22 ± 49.52***	0.016*
	Phe Ile (μg ml^{-1} OD_{600}^{-1})	0.86 ± 2.10	**85.07 ± 46.36**	0.001*
	Leu (μg ml^{-1} OD_{600}^{-1})	-	-	-
	Lys (μg ml^{-1} OD_{600}^{-1})	-	-	-
	Pro (μg ml^{-1} OD_{600}^{-1})	747.75 ± 120.88	728.90 ± 156.43	0.820

- Not detectible.
* Statistically significantly different between BW25113 and EJW3 (*p*-values < 0.05). Values bolded are significantly higher in EJW3. Values bolded and italicized are significantly lower in EJW3. *P*-values are calculated by using a two-tailed student's *t*-test with six biological replicates.

glutamic acid production under osmotic stress in both BW25113 and EJW3 strains. However, EJW3 appears to accumulate more glutamic acid intracellularly, as the intracellular level of glutamic acid was ~24% higher (*p*-value = 0.006), while the extracellular concentration was ~62% lower (*p*-value < 0.001) in EJW3 compared with BW25113. This suggests that EJW3 may be better at accumulating glutamic acid intracellularly under osmotic stress due to reduced export of the amino acid into the bulk medium. A similar trend was observed with arginine levels, with both strains producing more arginine in the presence of osmotic stress, and EJW3 maintaining ~56% higher intracellular arginine concentration (*p*-value < 0.001) compared with the wild-type (Table 2.11). It has also been reported that cold osmotic shock reduced the ability of *E. coli* to accumulate arginine (112). The ability of EJW3 to produce and accumulate more arginine than BW25113 may also contribute to its higher osmotic tolerance. Alanine is known to be an important osmolyte in many organisms (113), but thus far has not been

reported to serve as an osmolyte in *E. coli*. Our results showed that the production of alanine decreased under osmotic stress in both strains, but the intracellular concentration was ~86% higher in EJW3 than in BW25113 (*p*-value = 0.001). Though the production of alanine did not increase in response to osmotic stress, the data suggests that biosynthesis of this amino acid was not significantly inhibited in EJW3 in the presence of osmotic stress compared with BW25113. In addition to known amino acids that are perturbed by osmotic stress in bacteria, our results showed that the production of methionine, tyrosine, tryptophan, phenylalanine and isoleucine were also perturbed by osmotic stress and the *rpoC* mutation (Tables 2.10 and 2.11).

Trehalose is one of the most well-known osmoprotectants. Prior reports showed increased production of trehalose in response to osmotic stress to prevent water loss and to maintain the intracellular pressure of the cells (77, 86). We found that the intracellular concentration of trehalose under osmotic stress to be ~37% higher in EJW3 than BW25113 (*p*-value = 0.005), which may be another reason for the higher osmotic tolerance conferred by the *rpoC* mutation.

In addition to known osmoprotectants and amino acids, we also compared the intracellular and extracellular levels of organic acids between the two strains. The results showed a 30 - 40% higher extracellular acetic acid concentration in EJW3 cultures (*p*-value < 0.001) with or without osmotic stress challenge. However, EJW3 showed a ~40% lower intracellular acetic acid concentration in the absence of (*p*-value < 0.001), and a similar level of intracellular acetic acid concentration in the presence of (*p*-value = 0.137) hyperosmotic stress. Taking into account the relative total volume of extracellular

versus intracellular metabolites collected (a ratio of 100:1.5), the total amount of acetic acid produced by EJW3 (370 ± 20 μg ml $^{-1}$ OD_{600}^{-1}) was about 30% higher (p-value < 0.001) than that of BW25113 (290 ± 20 μg ml $^{-1}$ OD_{600}^{-1}). During growth on glucose as carbon source, the acetate secretion of *E. coli* has been shown to depend on growth rate (114, 115), thus the higher level of extracellular acetic acid in strain EJW3 may not be a direct consequence of the *rpoC* mutation but a side effect of the higher growth rate of the *rpoC* mutant under osmotic stress. However, it is possible that the higher acetic acid production may also contribute to the higher osmotic tolerance of EJW3.

2.4.4 Effect of acetic acid on osmotic stress tolerance

To determine the role acetic acid plays on osmotic tolerance, we compared the growth kinetics of BW25113 and EJW3 in the presence of 10 mM acetic acid in M9 with or without the supplementation of 0.6 M NaCl. In the absence of osmotic stress, the addition of 10 mM acetic acid showed no significant impact on the growth of either strain (Figure 2.7A). However, the addition of acetic acid significantly improved the growth of BW25113 but only had a moderate impact on the growth of EJW3 in the presence of NaCl challenge (Figure 2.7B). Under higher osmotic stress with 0.7 M NaCl, acetic acid addition still had a benefit on the growth of BW25113, and showed a moderate benefit on the growth of EJW3 (Figure 2.7C). Prior work has shown that moderate concentration of NaCl can protect *E. coli* from acetic acid toxicity (116); here we show that the cross tolerance is reciprocal, that moderate concentrations of acetic acid can also protect *E. coli* from NaCl stress. Interestingly, a statistically significant overlap ($p < 10^{-20}$, Fisher's exact test) between metabolites level changes in organic acid

and osmotolerant *E. coli* mutants has been detected using an updated version of the Resistome combined with a recent genome-wide survey of genotype-metabolite relationships (117, 118), providing additional evidence that the improved osmotic tolerance of EJW3 may also result from the higher production of acetic acid.

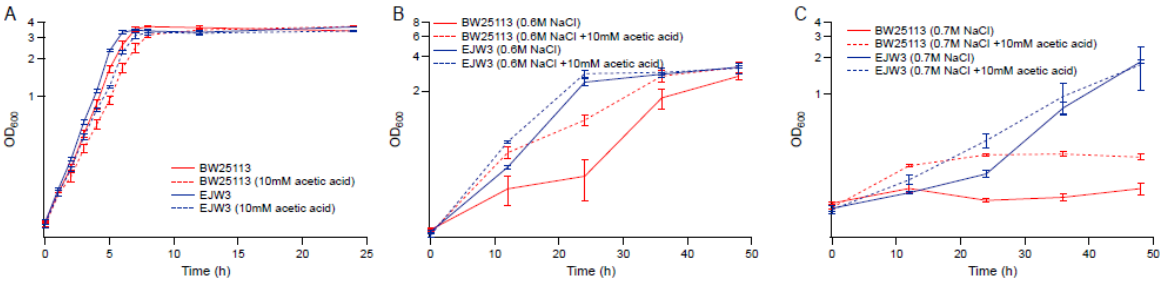

Figure 2.7. Effect of acetic acid addition on osmotic tolerance. (A) M9 only. (B) M9 supplemented with 0.6 M NaCl. (C) M9 supplemented with 0.7 M NaCl. Error bars are standard deviations.

2.4.5 Cell membrane damage analysis

As osmotic stress perturbs the membrane, propidium iodide (PI) assay was used to assess any differences in membrane perturbation between BW25113 and EJW3 in the presence of hyperosmotic stress in different growth phases. Propidium iodide is a fluorescent molecule that normally cannot penetrate into cells with intact membrane (119, 120); however, if the membrane integrity is compromised, the uptake of PI increases, and can be used to assess relative levels of membrane perturbation. In order to detect differences within a short-term challenge, a higher hyperosmotic stress with 0.7 M NaCl was used in this test. The membrane integrity of both strains was assessed after a

30-minute exposure to 1) M9 only, 2) M9 supplemented with 0.7 M NaCl, 3) M9 supplemented with 10 mM acetic acid, and 4) M9 supplemented with 0.7M NaCl and 10 mM acetic acid. Cells in either lag phase (OD_{600} ~ 0.1), exponential phase (OD_{600} ~ 0.8) or stationary phase (OD_{600} ~ 3.0) were tested. Low levels of PI staining were observed in both strains in lag phase and stationary phase cells with or without NaCl challenge (Figure 2.8), suggesting no major membrane perturbation by NaCl challenge during these non-growth phases. However, in exponential growing cells, BW25113 showed significantly higher PI staining than EJW3 (Figure 2.9) under 0.7 M NaCl challenge, suggesting a lower level of membrane perturbation in the *rpoC* mutant under hyperosmotic stress. While our data showed that addition of 10 mM acetic acid improved growth of BW25113 in hyperosmotic stress (Figure 2.7), PI staining data revealed that the addition of acetic acid conferred no benefit to the hyperosmotic stress induced membrane perturbation in either strain, which suggests that the benefit of acetic acid to osmotic tolerance is not related with membrane integrity.

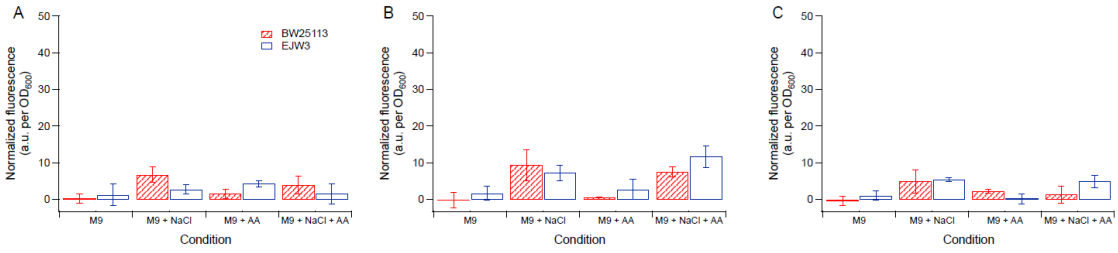

Figure 2.8. Membrane integrity assay using PI staining. (A) lag phase cells. (B) Early stationary phase cells. (C) Late stationary phase cells. NaCl: 0.7 M NaCl. AA: 10 mM acetic acid. Error bars are standard deviations.

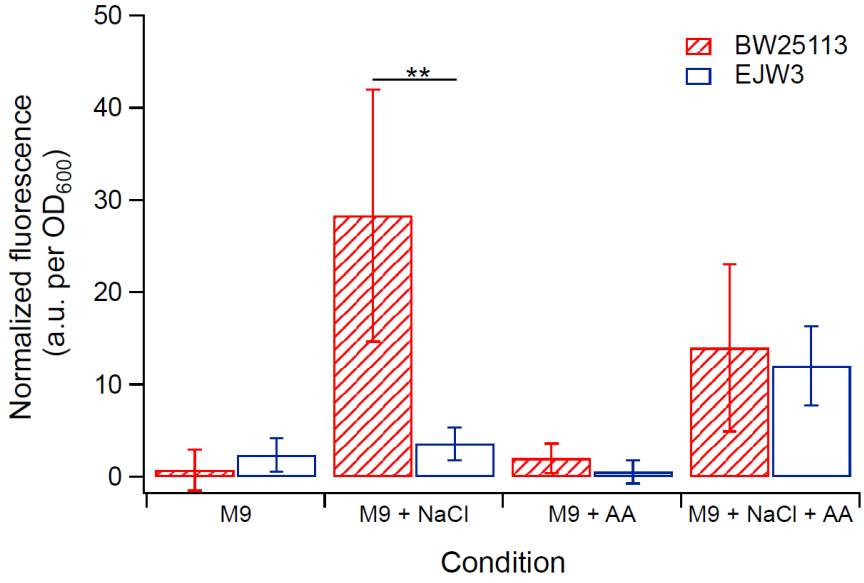

Figure 2.9. Membrane integrity assay using PI staining of exponential growing cells. NaCl: 0.7M NaCl. AA: 10 mM acetic acid. ** Statistically significantly different using 2 tailed student t-test (*p*-value < 0.005). Error bars are standard deviations.

2.4.6 Transcriptional profile analysis

RpoC is the β' subunit of the RNA polymerase complex, and plays roles in promoter recognition, sigma factor binding and ion chelation (121-123). The *rpoC* K370_A396dup mutation is located in domain 2 (amino acid residues 344 – 486) of the RpoC subunit (124), close to the clamp (amino acid residues 14 – 342) and the NADFDGD (amino acid residues 458 – 464) motif which is involved in Mg2+ binding (123, 125); thus this mutation in *rpoC* may impact transcriptional initiation and elongation (126, 127). Furthermore, the 3-D structure of the RNA polymerase places the K370_A396dup mutation in close proximity to the proposed binding site of guanosine tetraphosphate (ppGpp) (amino acid residues 362, 417) (128-130), which is a global regulator involved in stringent response (131). Thus, the *rpoC* K370_A396dup mutation

is expected to have global impact on the transcriptional regulation of the cell, therefore the transcriptional differences in BW25113 and EJW3 can potentially be used to identify new molecular mechanisms of osmotolerance in *E. coli*. Thus, we compared the transcriptional profiles between EJW3 and BW25113 in M9 with or without 0.6 M NaCl challenge to identify any transcriptional regulatory differences that may lead to new findings on molecular mechanisms for osmotic tolerance. The transcriptional data showed that most genes upregulated in strain EJW3 under osmotic stress are related with amino acids metabolism, which corroborated with our data from amino acid supplementation and metabolite analysis; and the genes downregulated are related with membrane composition and transporters.

Table 2.12 Genes selected for validation for their roles in osmotic tolerance.

ID	Function
b0260	CP4-6 prophage; putative S-methylmethionine transporter (*mmuP*)
b3939	cystathionine gamma-synthase, PLP-dependent (*metB*)
b4013	homoserine O-transsuccinylase (*metA*)
b3828	methionine biosynthesis regulon transcriptional regulator (*metR*)
b2942	S-adenosylmethionine synthetase (metK)
b2421	cysteine synthase B (O-acetylserine sulfhydrolase B) (*cysM*)
b2366	D-serine dehydratase (*dsdA*)
b4131	lysine decarboxylase, acid-inducible (*cadA*)
b0402	proline-specific permease (*proY*)
b1386	tyramine oxidase, copper-requiring (*tynA*)

Several genes upregulated in strain EJW3 under osmotic stress were chosen for further validation for their role in osmotic tolerance in the *rpoC* K370_A396dup background. The selected genes (Table 2.12) are involved in amino acids metabolism

and were chosen based on results from the amino acid supplementation and metabolite analyses. These genes were overexpressed in BW25113 by using clones from the ASKA collection (103), and the growth kinetics of the overexpression strains were compared against the control expressing the empty plasmid in M9 with or without NaCl supplementation. To avoid potential toxicity associated with high level gene expression, basal level expression (without IPTG induction) and a lower osmotic stress (0.55 M NaCl) were used. Among the genes tested, only the overexpression of *metK* and *mmuP* significantly improved the performance of BW25113 under osmotic stress. The overexpression of *metK* and *mmuP* did not impact the growth of BW25113 in the absence of hyperosmotic stress, but confer a benefit in the presence of 0.55 M NaCl (Figure 2.10). This suggests that the osmotic tolerance conferred by the *rpoC* mutation in BW25113 is partially due to the upregulation of *metK* and *mmuP*. *MetK* encodes the methionine adenosyltransferase that catalyzes the formation of the S-adenosylmethionine (AdoMet) (132), which is involved in many biological reactions. It plays important roles as donors of methyl, sulfur, and aminopropyl groups, thus is frequently involved in the regulation of gene expression, including genes involved in methionine metabolism (133-136), but was not known to be related with osmotolerance. *MmuP* is a transporter of S-methylmethionine, which can be used by *E. coli* as a source of methionine when externally provided (137). S-methylmethionine is widely produced by plants and can be used as an osmoprotectants in plants (138), but it has not been reported as an osmoprotecant in *E. coli*. As *E. coli* is not known to produce S-methylmethionine, nor did we supplement S-methylmethionine into the media, in this

case, the impact of *mmuP* on osmotic tolerance is not obvious. Stress response genes *bolA* and *hdeA* are downregulated in the BW25113 *rpoC* mutant, and the deletion of *bolA* and *hdeA* improved the growth of BW25113 in the presence of 0.6 M NaCl (Figure 2.11). BolA is a transcriptional regulator involved in regulating cell morphology related genes, and is known to be involved in general stress response and has been reported to be overexpressed under osmotic stress (139); however, the mechanism of how it is involved in osmotic tolerance remains unclear. HdeA is a periplasmic acid stress chaperone which plays a role in resistance to low pH (140, 141), and it is not known to be related with osmotic tolerance.

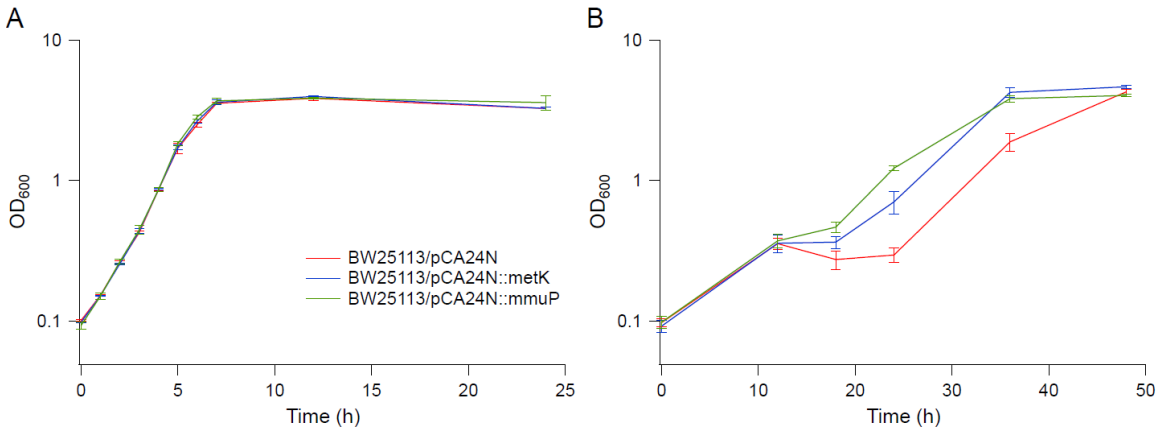

Figure 2.10. Growth kinetics of overexpression strains in BW25113 background. (A) M9. (B) M9 supplemented with 0.55 M NaCl. Error bars are standard deviations.

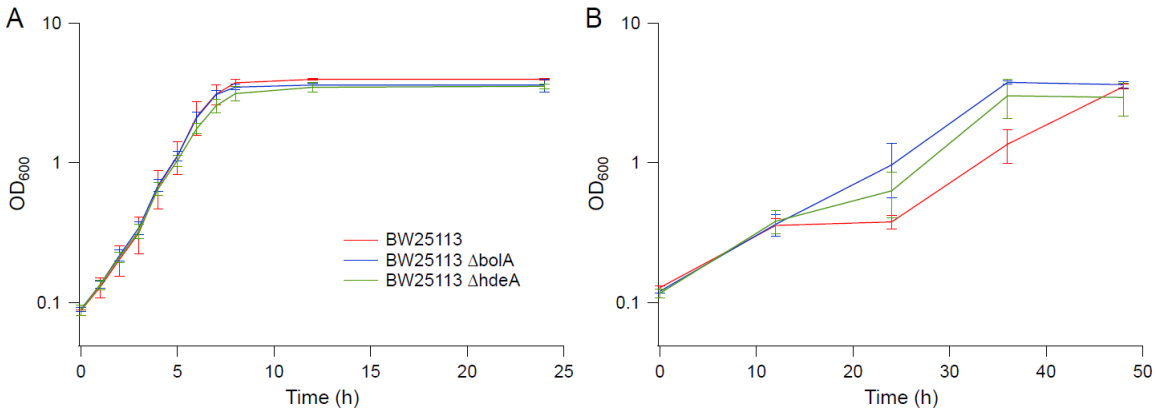

Figure 2.11. Growth kinetics of knockout strains in BW25113 background. (A) M9. (B) M9 supplemented with 0.6 M NaCl. Error bars are standard deviations.

As stated previously, the K370_A396dup mutation is close to the proposed ppGpp binding site on RpoC (128-130). PpGpp is known to be involved in stringent response upon nutrition starvation or other stress conditions, and has global impact on gene expression (131, 142). It regulates transcriptional initiation directly by binding to an interface between the β′ and ω subunit of the RNA polymerase, or indirectly by altering sigma factor availability, and it also indirectly affects translation and DNA replication (142, 143). It has recently been reported that supplementation of serine hydroxamate (SHX), which induces production of ppGpp, increased osmotic tolerance of ppGpp-proficient *E. coli*, which was not observed in ppGpp-deficient cells, demonstrating that ppGpp is involved in osmotic tolerance (144). Furthermore, an RpoC Δ312-315 mutation was previously found to suppress the ppGpp deficiency phenotypes (145). Since the *rpoC* K370_A396dup mutation falls outside the amino acid residues known to impact RpoC interaction with ppGpp, any relationship between the *rpoC* K370_A396dup mutation and ppGpp remains to be investigated.

2.4.7 Impact of the rpoC mutation in MG1655

To determine whether the *rpoC* mutation exhibits a similar benefit to osmotic stress tolerance in other *E. coli* K-12 strains, the *rpoC* mutation was reconstructed in MG1655 to generate strain EYG1. The osmotic tolerance of MG1655 and EYG1 were assessed in M9 with or without challenge with 0.6 M NaCl (shown in Figure 2.12A and B, respectively). In contrast to BW25113, the *rpoC* K370_A396dup mutation did not confer a benefit to MG1655 under osmotic stress. Though the growth of both MG1655 and EYG1 were slower than BW25113 in M9 in the absence of hyperosmotic stress (Figure 2.12A), their growths were nearly identical to EJW3 under osmotic stress (Figure 2.12B), suggesting that the inherent tolerance of MG1655 to NaCl was higher than BW25113, and led us to use a higher NaCl concentration with subsequent assays with MG1655.

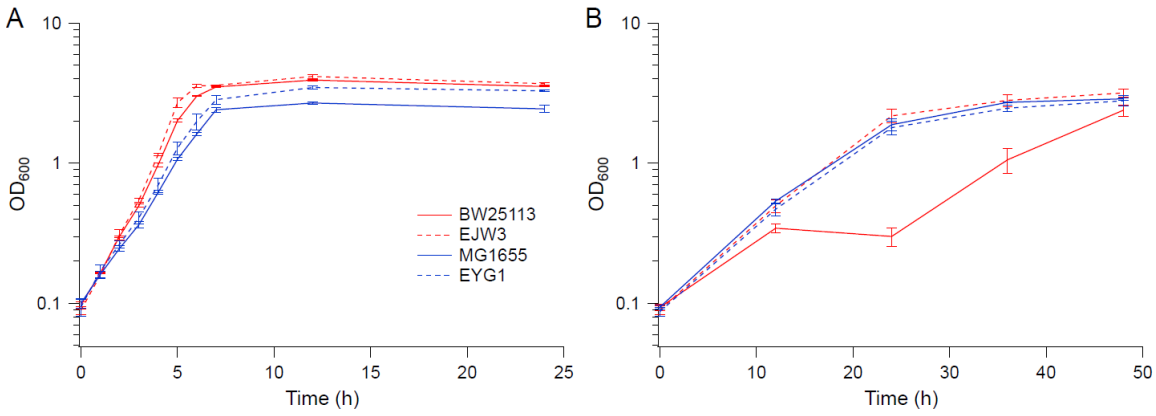

Figure 2.12. Growth kinetics of MG1655, EYG1, BW25113 and EJW3. (A) M9 (B) M9 supplemented with 0.6 M NaCl. Error bars are standard deviations.

As we found tryptophan supplementations to benefit BW25113 in the presence of hyperosmotic challenge, we also assessed its potential benefit in MG1655. In the presence of 0.7 M NaCl, addition of tryptophan to the medium increased the osmotic tolerance of MG1655 and EYG1 (Figure 2.13A). The deletion of *bolA* also improved the growth of MG1655 under osmotic stress with 0.7 M NaCl (Figure 2.13B, C), which suggests that although the impact of the *rpoC* mutation on osmotic tolerance appears to be strain specific, some of the findings based on BW25113 also applies to MG1655.

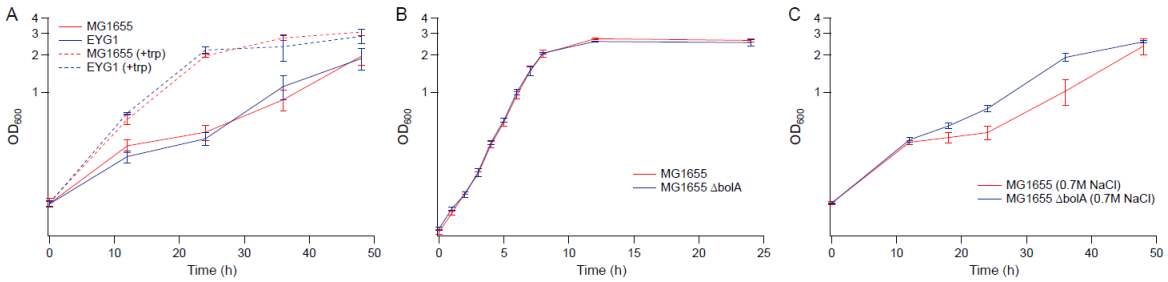

Figure 2.13. Growth kinetics of MG1655, EYG1 and the knockout strains in MG1655 background. (A) M9 supplemented with 0.7 M NaCl with and without 50 µg ml^{-1} tryptophan (B) M9 (C) M9 supplemented with 0.7 M NaCl. Error bars are standard deviations.

We also compared the transcriptional profiles between MG1655 and EYG1 (MG1655 background) in M9 with or without 0.6 M NaCl challenge to identify any transcriptional regulatory differences caused by the *rpoC* mutation between MG1655 and BW25113 background. The microarray data showed that the *rpoC* mutation caused significantly different transcriptional perturbation between these two strain backgrounds, with few similarities shared between strains and conditions. As mentioned previously,

most genes upregulated in strain EJW3 under osmotic stress are related with amino acids metabolism, however, in EYG1 (MG1655 background), most amino acids metabolism and transport related genes perturbed by the *rpoC* mutation were downregulated (Table 2.13). This difference between strains EJW3 and EYG1 likely explains the different physiological impacts of the *rpoC* mutation on osmotic tolerance between BW25113 and MG1655.

Table 2.13 Gene ontology analysis in M9 supplemented with 0.6 M NaCl.

	Category	Term	Count	%	*p*-value
Upregulated (BW25113)	GOTERM_BP_DIRECT	GO:0009086~methionine biosynthetic process	4	5.405	0.001
	UP_KEYWORDS	Methionine biosynthesis	4	5.405	0.002
	UP_KEYWORDS	Pyridoxal phosphate	6	8.108	0.004
Downregulated (BW25113)	UP_KEYWORDS	Transmembrane helix	21	41.176	0.001
	UP_KEYWORDS	Cell membrane	23	45.098	0.001
	UP_SEQ_FEATURE	transmembrane region	21	41.176	0.001
	UP_KEYWORDS	Transmembrane	21	41.176	0.001
	UP_SEQ_FEATURE	topological domain:Cytoplasmic	16	31.373	0.001
	UP_SEQ_FEATURE	topological domain:Periplasmic	16	31.373	0.002
	UP_KEYWORDS	Membrane	23	45.098	0.004
	UP_KEYWORDS	Cell inner membrane	19	37.255	0.005
	COG_ONTOLOGY	Inorganic ion transport and metabolism	4	7.843	0.006

Table 2.13 Continued

	Category	Term	Count	%	*p*-value
Downregulated (BW25113)	GOTERM_BP_DIRECT	GO:0006814~sodium ion transport	3	5.882	0.009
	GOTERM_CC_DIRECT	GO:0005886~plasma membrane	21	41.176	0.012
Upregulated (MG1655)	KEGG_PATHWAY	eco00780:Biotin metabolism	4	3.636	0.001
Downregulated (MG1655)	KEGG_PATHWAY	eco00190:Oxidative phosphorylation	19	13.571	0.000
	UP_KEYWORDS	Quinone	10	7.143	0.000
	UP_KEYWORDS	Ubiquinone	10	7.143	0.000
	GOTERM_CC_DIRECT	GO:0045272~plasma membrane respiratory chain complex I	10	7.143	0.000
	GOTERM_CC_DIRECT	GO:0030964~NADH dehydrogenase complex	10	7.143	0.000
	GOTERM_BP_DIRECT	GO:0009060~aerobic respiration	11	7.857	0.000
	GOTERM_MF_DIRECT	GO:0048038~quinone binding	9	6.429	0.000
	GOTERM_MF_DIRECT	GO:0003954~NADH dehydrogenase activity	10	7.143	0.000
	UP_KEYWORDS	Ligase	15	10.714	0.000
	GOTERM_MF_DIRECT	GO:0008137~NADH dehydrogenase (ubiquinone) activity	8	5.714	0.000
	UP_KEYWORDS	Oxidoreductase	28	20	0.000
	UP_KEYWORDS	Enterobactin biosynthesis	5	3.571	0.000
	GOTERM_BP_DIRECT	GO:0006099~tricarboxylic acid cycle	8	5.714	0.000
	UP_KEYWORDS	Tricarboxylic acid cycle	7	5	0.000
	KEGG_PATHWAY	eco00020:Citrate cycle (TCA cycle)	9	6.429	0.000

Table 2.13 Continued

	Category	Term	Count	%	*p*-value
Downregulated (MG1655)	UP_KEYWORDS	Transport	43	30.714	0.000
	GOTERM_BP_DIRECT	GO:0009239~enterobactin biosynthetic process	5	3.571	0.000
	GOTERM_BP_DIRECT	GO:0042773~ATP synthesis coupled electron transport	5	3.571	0.000
	GOTERM_BP_DIRECT	GO:0015990~electron transport coupled proton transport	5	3.571	0.000
	KEGG_PATHWAY	eco02040:Flagellar assembly	10	7.143	0.000
	KEGG_PATHWAY	eco01053:Biosynthesis of siderophore group nonribosomal peptides	5	3.571	0.000
	UP_KEYWORDS	Cell inner membrane	49	35	0.000
	KEGG_PATHWAY	eco00250:Alanine, aspartate and glutamate metabolism	9	6.429	0.000
	UP_KEYWORDS	Pyrimidine biosynthesis	5	3.571	0.000
	GOTERM_MF_DIRECT	GO:0030976~thiamine pyrophosphate binding	5	3.571	0.000
	UP_KEYWORDS	Cell membrane	54	38.571	0.000
	UP_KEYWORDS	Membrane	58	41.429	0.000
	UP_KEYWORDS	NAD	14	10	0.001
	GOTERM_MF_DIRECT	GO:0015421~oligopeptide-transporting ATPase activity	4	2.857	0.001
	GOTERM_BP_DIRECT	GO:0044205~'de novo' UMP biosynthetic process	4	2.857	0.002
	UP_KEYWORDS	Bacterial flagellum biogenesis	5	3.571	0.002
	UP_KEYWORDS	Purine biosynthesis	5	3.571	0.002

Table 2.13 Continued

	Category	Term	Count	%	*p*-value
Downregulated (MG1655)	UP_KEYWORDS	Nucleotide-binding	29	20.714	0.002
	KEGG_PATHWAY	eco01100:Metabolic pathways	55	39.286	0.003
	GOTERM_BP_DIRECT	GO:0019646~aerobic electron transport chain	4	2.857	0.004
	UP_KEYWORDS	Bacterial flagellum	5	3.571	0.004
	GOTERM_MF_DIRECT	GO:0042936~dipeptide transporter activity	4	2.857	0.004
	UP_KEYWORDS	ATP-binding	25	17.857	0.004
	GOTERM_MF_DIRECT	GO:0047527~2,3-dihydroxybenzoate-serine ligase activity	3	2.143	0.004
	GOTERM_BP_DIRECT	GO:0044781~bacterial-type flagellum organization	4	2.857	0.005
	GOTERM_BP_DIRECT	GO:0042938~dipeptide transport	4	2.857	0.005
	UP_KEYWORDS	Thiamine pyrophosphate	4	2.857	0.006
	UP_KEYWORDS	Glutamine amidotransferase	4	2.857	0.006
	GOTERM_CC_DIRECT	GO:0009424~bacterial-type flagellum hook	4	2.857	0.006
	UP_SEQ_FEATURE	topological domain:Cytoplasmic	31	22.143	0.007
	UP_SEQ_FEATURE	topological domain:Periplasmic	31	22.143	0.007
	GOTERM_BP_DIRECT	GO:0015796~galactitol transport	3	2.143	0.008
	GOTERM_MF_DIRECT	GO:0090584~protein-phosphocysteine-galactitol-phosphotransferase system transporter activity	3	2.143	0.008

2.5 Conclusion

In this work, we confirmed that the in-frame, 84bp duplication in *rpoC* (K370_A396dup) does indeed contribute to osmotic tolerance of *E. coli*. The *rpoC* K370_A396dup mutation was reconstructed in the BW25113 background (EJW3), and the *rpoC* mutant performed much better than the wild-type strain under osmotic stress. From individual amino acid supplementation studies, we found several additional amino acids (*e.g.* tryptophan, phenylalanine, *etc*.) to play a role in improving osmotic tolerance in *E. coli*. Results from metabolite analysis revealed differences in intracellular and extracellular metabolites between the wild-type and *rpoC* mutant, suggesting alterations in metabolism may be partially responsible for the enhanced hyperosmotic tolerance in the mutant. Compared with the wild-type strain, EJW3 produced and accumulated more known osmoprotectants (trehalose, proline, and glutamic acid) in response to hyperosmotic stress. In addition to known osmolytes, EJW3 also produce approximately 30% more acetic acid than BW25113. Subsequent supplementation studies confirmed that the addition of moderate concentration of acetic acid helps to improve the performance of BW25113 under hyperosmotic stress; which supported the theory that the higher production of acetic acid is likely related with the osmotic tolerance conferred by the *rpoC* (K370_A396dup) mutation. Membrane damage analysis using PI staining showed a lower effect on membrane integrity in the presence of hyperosmotic stress in *rpoC* mutants that are actively growing (in exponential growth phase). Transcriptional analysis demonstrated that the *rpoC* mutation indeed impacted relative transcript abundance of genes related with amino acids metabolism, including *metK* and *mmuP*.

When overexpressed in BW25113, *metK* and *mmuP* conferred enhanced osmotic tolerance, providing further validation that methionine metabolism is involved in enhanced hyperosmotic tolerance in the *rpoC* mutant. Deletion of stress response genes *bolA* and *hdeA* also improved growth of BW25113 under osmotic stress, and the benefit of *bolA* deletion on osmotic tolerance also applies to MG1655. Other genes perturbed by the *rpoC* mutation in BW25113 and the synergistic effects of those genes remain to be investigated. In conclusion, the work demonstrated that the impacts of this particular *rpoC* mutation on *E. coli* metabolism and membrane integrity related with osmotic tolerance, and although the impacts of *rpoC* mutation appeared to be strain dependent, some of the findings in BW25113 also apply to another K-12 strain. Results from this work can help to identify targets for metabolic engineering of *E. coli* for enhanced tolerance to alternative feedstocks and water sources.

3 PRODUCTION OF CAROTENOIDS BY *SACCHAROMYCES CEREVISIAE* IN SEAWATER MEDIA

3.1 Summary

The use of seawater has been reported to improve carotenoids production of some native producers. Thus, we hypothesized that seawater may also affect carotenoids production of a previously developed *S. cerevisiae* carotenoids hyperproducer SM14. When using reduced nutrient media in synthetic seawater, the carotenoids production of SM14 increased approximately 2-fold compared with the production in fresh water. We found that this improvement was partially due to the NaCl present in the synthetic seawater. The combination of synthetic seawater with higher carbon-to-nitrogen ratio (C:N=50) further improved carotenoids production. These results demonstrated the potential benefit of using seawater to improve the production of carotenoids in *S. cerevisiae*.

3.2 Introduction

Carotenoids is a large class of pigmented compounds naturally produced by plants, algae, and some fungi and bacteria (146-151). In the native organisms, these compounds serve as natural colorants, photoprotective agents and antioxidants (146, 147, 152-154). Carotenoids also play roles as vitamin A precursors, antioxidants and antimicrobials in animals (152, 155, 156). Thus, these compounds have potential applications in the food, cosmetics, and health industries (157-162). Currently, most of

the carotenoids produced in industry are extracted from natural sources such as plants and algae, or chemically synthesized. However, the structural complexity of most carotenoids make them difficult to be synthesized chemically, and the solvent-based chemical extraction from plants and algae faces challenges due to uncontrollable feedstock availability (153, 163). Thus, there have been extensive efforts to engineer microbial hosts for their production (164-173). While the majority of prior metabolic engineering efforts have focused on pathway engineering, in our prior work, we developed a novel evolution engineering approach based on the antioxidant potential of β-carotene and successfully generated carotenoids hyper-producing strains of *Saccharomyces cerevisiae* (174). In subsequent work, we optimized bioreactor conditions for production using fresh water (175). However, industrial fermentation requires large amount of water, which generates pressures on fresh water (176). Using alternative water sources such as seawater in industrial fermentation will help to reduce freshwater usage in bio-based production of chemicals (177). Prior work has shown the use of seawater media to be promising for ethanol fermentation by *Saccharomyces cerevisiae* (177), and seawater media has also been used to improve carotenoids production in *Rhodotorula glutinis* (178). Therefore, seawater may also have the potential to improve carotenoids production in *Saccharomyces cerevisiae*. In this work, we analyzed the impact of using seawater and reduced nutrients on the carotenoids productivity of a hyper-producer (SM14) generated from our prior work, and showed that the carotenoids production of the engineered non-native producer can also been improved by using seawater media.

3.3 Materials and methods

3.3.1 Strains and growth conditions

Saccharomyces cerevisiae strains SM14, SM11, SM12, SM13, SM22, SM24 and YLH2 (174) were used in this study (Table 3.1). Unless otherwise noted, strains were cultured in test tubes with 3 ml yeast nitrogen base (YNB, 1.7 g L^{-1}) or 1/10× yeast nitrogen base (1/10× YNB, 0.17 g L^{-1}) media supplemented with 5 g L^{-1} of ammonium sulfate and 20 g L^{-1} of glucose made with either freshwater or synthetic seawater (RICCA) at 30ºC. All experiments were performed in triple replicates and statistical tests for significance were determined via 2-tailed student *t*-test.

Table 3.1 Strains used in this study.

Name	Description	Reference
YLH2	Unevolved ancestral strain	(174)
SM14	Isolated hyper-producer from evolved population 1	(174)
SM11	Isolated hyper-producer from evolved population 1	(174)
SM12	Isolated hyper-producer from evolved population 1	(174)
SM13	Isolated hyper-producer from evolved population 1	(174)
SM22	Isolated hyper-producer from evolved population 2	(174)
SM24	Isolated hyper-producer from evolved population 2	(174)

3.3.2 Carotenoids quantification

Carotenoids quantification was performed as previously described (174, 175). Briefly, 250 µl or 500 µl of culture were transferred to a 2 ml screw-capped microcentrifuge tube and the cells were collected by centrifugation at 21,300× g for 2 minutes. Then the supernatant was vacuum aspirated, and approximately 250 µl of 425 - 600 µm acid-washed glass beads (Sigma) and dodecane (1 ml) was added to the

microcentrifuge tube to extract the carotenoids. The pelleted cells were lysed using Disruptor Genie Cell Disruptor (Scientific Industries) twice for 6 minutes each to ensure maximum cell disruption and carotenoids recovery, followed by centrifugation at 21,300× g for 2 minutes to separate cell debris and glass beads. 200 µl of the supernatant was transferred to a 96-well black-wall clear-bottom plate (Greiner) for quantification and scanned by a microplate reader (TECAN Infinites M200). Intracellular β-carotene was quantified at absorbance wavelength of OD_{454}. A standard curve for β-carotene quantification was generated using commercially available β-carotene (Enzo Life Sciences) at OD_{454}. β-carotene was also quantified using high-performance liquid chromatography (HPLC, Agilent Technologies, 1260 Infinity, Santa Clara, CA) and Agilent ZOBAX Eclipse Plus C18 column (4.6 × 100 mm, 3.5-Micron) run at 40°C with acetonitrile-methanol-isopropanol (50: 30: 20, v: v: v) as the mobile phase. The flow rate of mobile phase was set at 1 ml min^{-1}, and carotenoids were detected using a UV detector at 450 nm (179). The quantification by HPLC and absorbance were compared and were found to correlate well. The simpler spectrophotomeric assay was used for carotenoids quantification in this work.

3.3.3 Lipid quantification

The total lipid of *S. cerevisiae* was extracted in the form of fatty acid methyl ester (FAME) using the sulfuric acid–methanol method and quantified by GC-MS as previously described (180). Briefly, cells in 25 ml cultures were harvested by centrifugation (4,470× g) at 4 ºC for 5 minutes. Cell pellets were transferred in 2 ml screw-capped microcentrifuge tube, approximately 250 µl of 425 - 600µm acid-washed

glass beads (Sigma) and methanol (1 ml) was added to the collection tube to extract total lipids. 10 µl of 10 mg ml^{-1} heptadecanoic acid (Alfa Aesar) was also added to the collection tube as internal standards. The cells were lysed using the Disruptor Genie Cell Disruptor (Scientific Industries) twice for 6 minutes each time. The lysed cells (in 1 ml methanol) was transferred to a 50 ml centrifuge tube and 9 ml methanol was added to the tube. The mixture was incubated in a 65°C water bath for 30 minutes, vortexed for 5 seconds every 10 minutes. Then 0.5 ml of 10 N KOH was added to the tube, vortexed vigorously for about 1 minute, incubated at 65°C for 2 hours, and vortexed for 5 seconds every hour. After cooling to room temperature in a cold tap water bath, 0.5 ml of concentrated H_2SO_4 (18.4 M) was added to the tube and the same 65°C incubation was repeated. After cooling to room temperature, 4 ml hexane was added to the tube to extract the lipids, vortexed for 1 minute, then centrifuged at 1,351× g for 5 minutes. The top hexane layer was then transferred to a clean 13 × 100 mm glass tube. This extraction step was repeated with another 4 ml hexane and the top hexane layer was transferred to the same glass tube. The extracted lipids were dried under nitrogen gas, and resuspended in 0.5 ml hexane for lipid composition analysis.

The lipid composition was analyzed by GC/MS using an SHIMADZU-QP2010 SE GC-MS (SHIMADZU CORPORATION, Japan) equipped with a ZB-5MSi column (thickness 0.25 µm; length 30 m; diameter 0.25 mm). The injection port was kept at 280°C and the MS transfer line was set to 100°C. The GC oven temperature was set at 40°C initially for 0.5 minute, increased to 110°C at a speed of 5°C min^{-1}, and then increased from 110°C to 300°C at a speed of 20°C min^{-1} for a total run time of 24

minutes. The raw chromatography and mass spectral data were processed with the software Enhanced ChemStation (Agilent Technologies, Santa Clara, CA), and the quantity of the specific lipid molecules was analyzed by the peak area.

3.4 Results

3.4.1 Impact of YNB concentration on β-carotene production of SM14

Seawater contains some trace elements (Na^+, Mg^{2+}, K^+, Ca^{2+}, Cl^-, SO_4^{2-}) that are present in yeast nitrogen base (YNB), thus, we hypothesized that using seawater may reduce the requirement of YNB. So, we compared the β-carotene production of SM14 with different concentration of YNB (1×, 0.7×, 0.4×, 0.1×) in fresh water and seawater. As show in Figure 3.1, biomass formation and β-carotene titer decreased as YNB concentration decreased, and the β-carotene yields are not significantly different in fresh water and seawater with 1×, 0.7×, and 0.4× YNB. However, with 0.1× YNB, the biomass formation, β-carotene titer and yield in seawater were significantly higher than that in fresh water (p-value < 0.01). Furthermore, the β-carotene yield in seawater with 0.1× YNB was significantly higher than that with higher YNB concentrations (p-value < 0.01), which indicated that SM14 produced and accumulated more carotenoids in seawater under reduced nutrient conditions. Since the difference of β-carotene production in fresh water and seawater are more obvious with lower YNB concentration, subsequent experiments were conducted with 0.1× YNB.

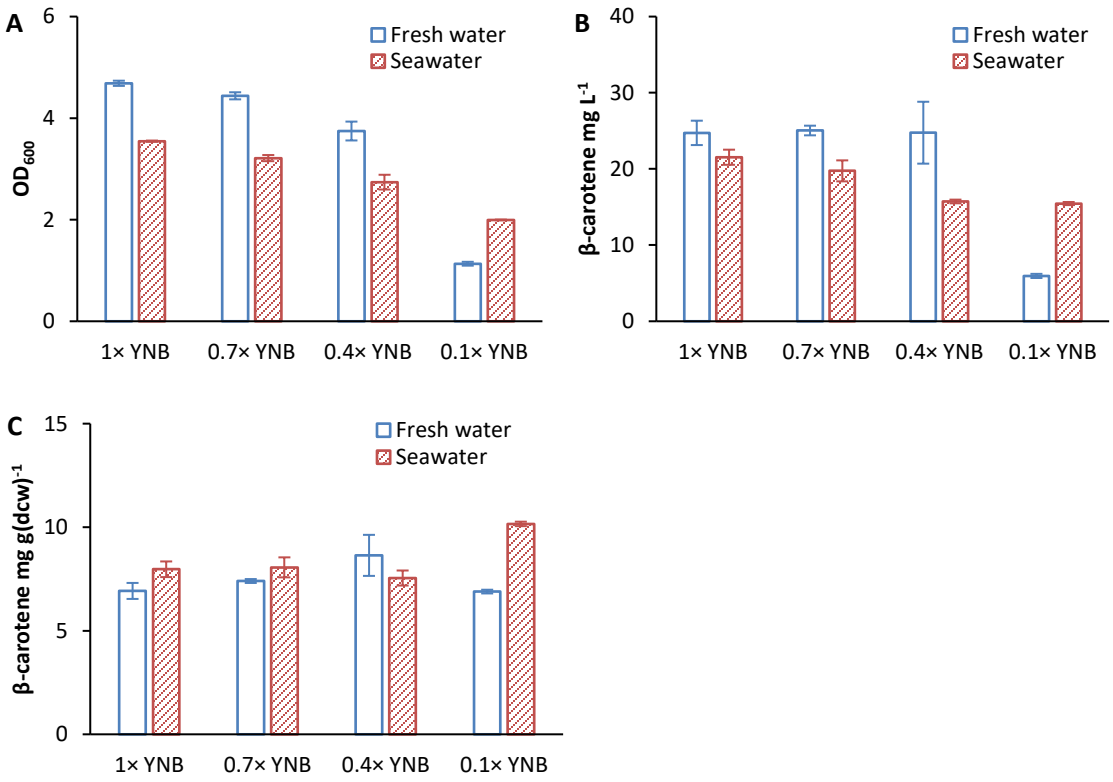

Figure 3.1. Biomass and β-carotene production of SM14 with different concentration of YNB in fresh water and seawater after 72 hours incubation. (A) Biomass (OD_{600}). (B) Titer. (C) Yield. Error bars are standard deviations.

3.4.2 Impact of water sources on β-carotene production of SM14

For reduced nutrient condition, we first tested the impact of water source on the carotenoids production of SM14 in 20 ml media using either fresh water, 1/3× seawater (water: synthetic seawater = 2:1), or seawater in 125 ml flasks. As show in Figure 3.2A, the growth of SM14 in seawater was significantly impacted, with a 24 hour longer lag phase; however, the culture reached a slightly higher final cell density compare with that in freshwater. Interestingly, no growth inhibition and a higher final cell density were observed in 1/3× seawater.

The β-carotene production of strain SM14 in seawater and 1/3× seawater were significantly increased compared with that in fresh water (Figure 3.2B). The β-carotene yield observed in fresh water peaked at 8.25 ± 0.51 mg g^{-1} dry cell weight (dcw) after 72 hours. On the other hand, the β-carotene production in seawater and 1/3× seawater continued to increase and reached 13.70 ± 1.29 mg $g(dcw)^{-1}$ and 17.37± 1.12 mg $g(dcw)^{-1}$, respectively, after 120 hours.

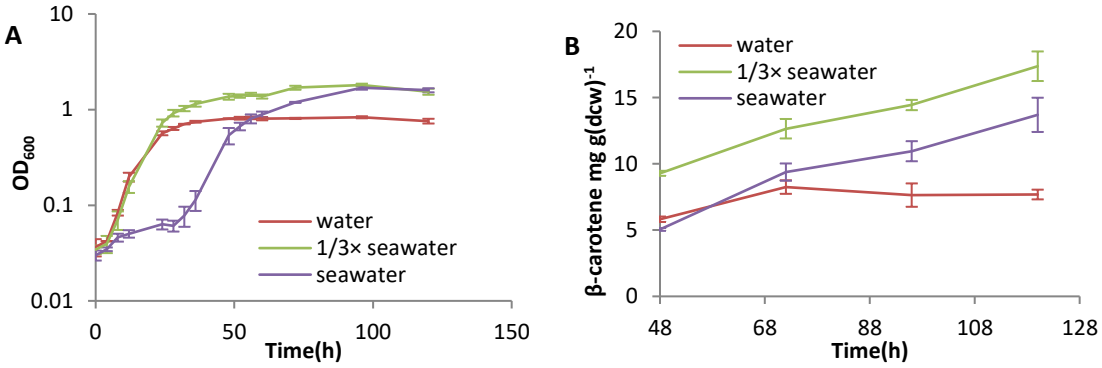

Figure 3.2. Growth kinetics of SM14 in 1/10× YNB made with water, 1/3× seawater, and seawater. (A) growth kinetics. (B) β-carotene production.

3.4.3 Impacts of NaCl on β-carotene production of SM14

The main component present in seawater is likely NaCl (the synthetic seawater contains ~0.41 M NaCl), thus, the role NaCl plays in the differences in growth and carotenoids production between seawater and fresh water was investigated. It has been reported that the addition of NaCl increased carotenoids production in some native producers (181-185). Thus, we hypothesized that NaCl present in seawater affected the carotenoids production in SM14, and compared the carotenoids production of SM14

fresh water media supplemented with varying concentrations of additional NaCl (No NaCl, 0.05 M, 0.137 M, 0.2 M, 0.3 M, 0.41 M and 0.5 M NaCl). All concentrations of NaCl tested significantly improved carotenoids production of SM14 in the nutrient reduced media after 72 hours incubation (p-value < 0.05). However, the growth rate and final biomass decreased as the concentration of NaCl increased (Figure 3.3), suggesting that while NaCl had a detrimental impact on cell growth, it had a positive impact on beta-carotene production.

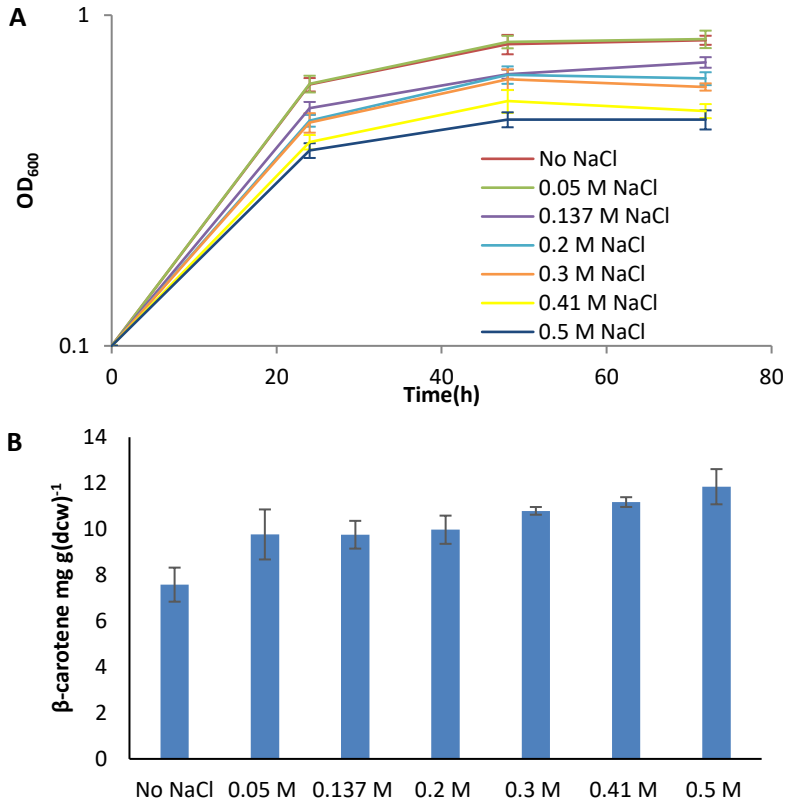

Figure 3.3. Growth kinetics and β-carotene production of SM14 in 1/10× YNB in fresh water with addition of different concentration of NaCl. (A) growth kinetics. (B) β-carotene production after 72 hours. Error bars are standard deviations.

3.4.4 Impacts of pH on carotenoids production of SM14 in reduced nutrient condition

The pH of the nutrient media differs depending on whether seawater or freshwater is used. The pH of the 0.1× YNB media in fresh water, 1/3× seawater, and seawater are ~5.2, 6.5, and 7.0 respectively. It has been reported that carotenoids production can be impacted by pH (175, 182). Thus, the improved carotenoids production in seawater may related with hither pH. So, we compared the carotenoids production in fresh water with pH ~5.2, 6.5, and 7.0. However, we observed no significant difference on carotenoids production after 120 hours when the initial pH of the reduced nutrient media in fresh water was varied between ~5.2, 6.5 and 7.0 (Figure 3.4A), and the pH decreased to ~4.0 in all conditions before reaching stationary phase (Figure. 3.4B) when carotenoids started to accumulate in cells.

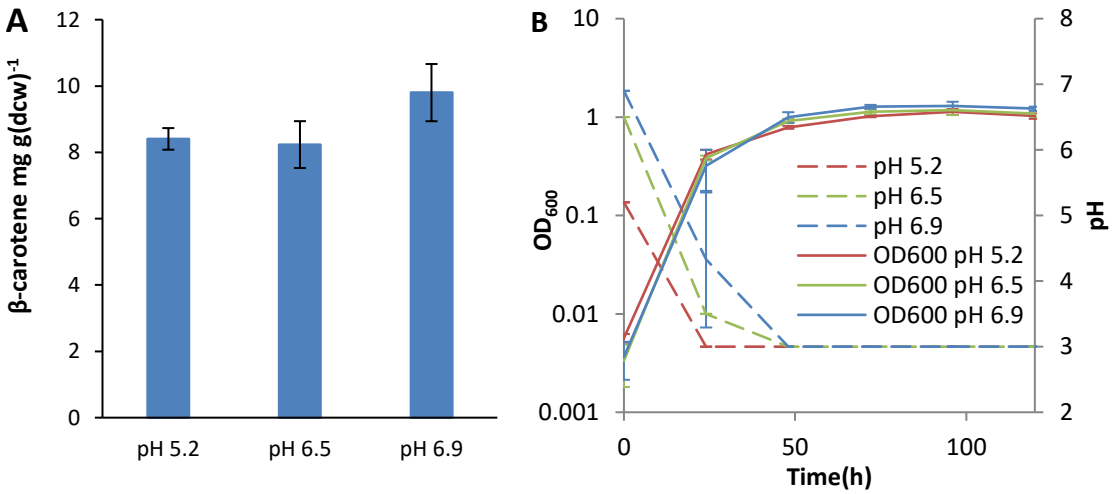

Figure 3.4. β-carotene production after 120 hours (A), growth and pH change (B) of SM14 in fresh water with different initial pH.

3.4.5 Impact of carbon-to-nitrogen ratio (C:N) on carotenoids production of SM14

We had previously demonstrated that increasing carbon to nitrogen (C:N) ratio from 8.8 to 50 improved carotenoids production of engineered yeast (including SM14) in bioreactors (175). To determine if increasing the C:N ratio also increases carotenoids production in reduced nutrient conditions, we compared the carotenoids production of SM14 in reduced YNB media with normal C:N ratio (8.8) and increased C:N ratio (50) in fresh water, 1/3× seawater and seawater (Figure 3.5). The results showed that after 72 hours incubation in all water sources, C:N=50 significantly increased carotenoids production of SM14 comparing with C:N=8.8 (p-value < 0.05), which indicates that increasing C:N ratio can further improve carotenoids production of SM14 in seawater.

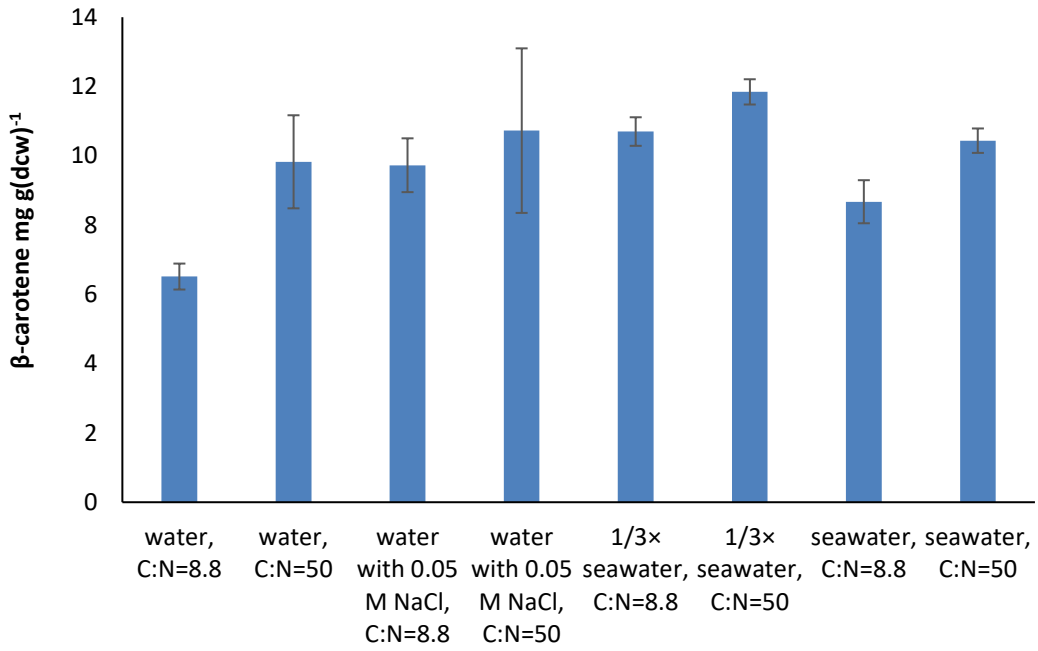

Figure 3.5. β-carotene production of SM14 in different conditions after 72 hours. Error bars are standard deviations.

3.4.6 Lipid production and composition

Carotenoids are hydrophobic chemicals, and our previous work showed that the improved carotenoids production by increasing C:N ratio to 50 was related with increased total lipid (175), thus, we hypothesized that the higher carotenoids production in seawater or by the addition of NaCl may also related with lipid production or composition. In order to validate this hypothesis, we analyzed total lipid production and composition of SM14 in reduced nutrient media with different water sources and different C:N ratios, and fresh water media with normal C:N ratio (C:N = 8.8) was used as the control condition. Samples in fresh water media were collected after 72 hours incubation and samples in seawater media were collected after 120 hours incubation when β-carotene production reached the highest level based on previous results (Figure 3.2). As show in Figure 3.6, increasing C:N ratio in water and in water with 0.05 M NaCl have significantly higher lipid titer, yield and ratio of unsaturated to saturated fatty acids than that in the control condition (p-value < 0.05). This result indicated that the higher carotenoids production in these conditions are related with the higher production of total lipids or the increased unsaturation of fatty acid caused by addition of NaCl or increasing C:N ratio. In seawater, increased C:N ration did not impact the lipid production or fatty acid saturation (Figure 3.7). Corresponded with β-carotene production (Figure 3.5), the total lipid yield and titer are higher in 1/3× seawater than in seawater (p-value < 0.05), which further proved that β-carotene production is related with lipid content.

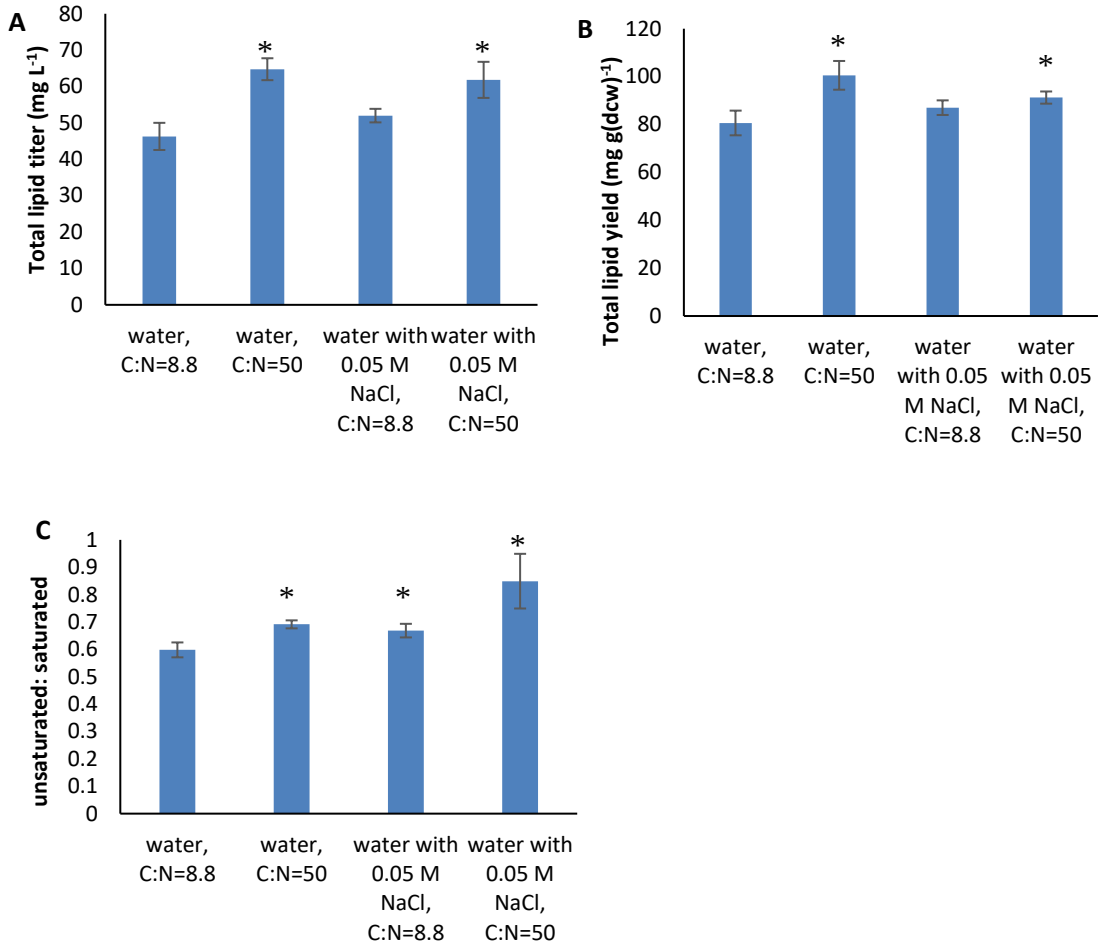

Figure 3.6. Total lipid titer (A), yield (B) and ratio of unsaturated fatty acids to saturated fatty acids (C) of SM14 in water. Error bars are standard deviations. * Statistically significantly different from data of water C:N=8.8 (p-value < 0.05).

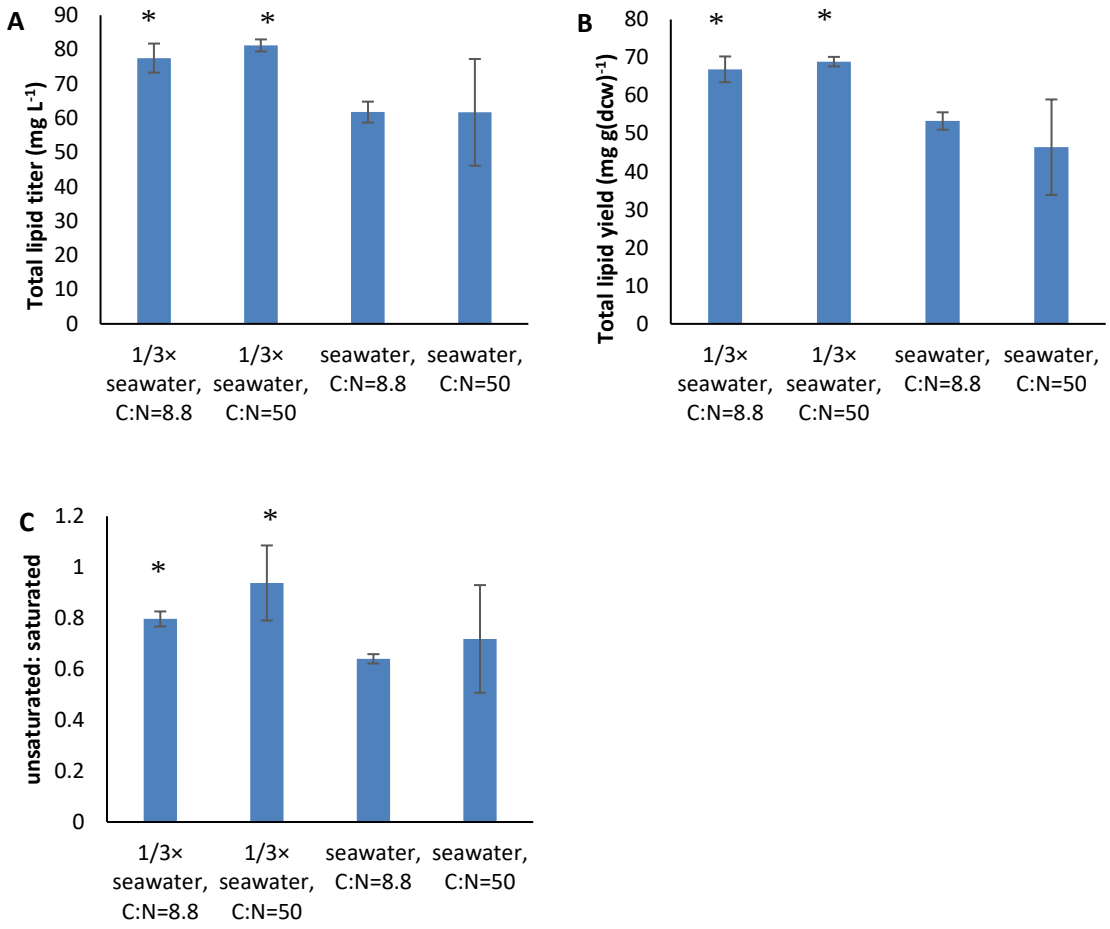

Figure 3.7. Total lipid titer (A), yield (B) and ratio of unsaturated fatty acids to saturated fatty acids (C) of SM14 in seawater. Error bars are standard deviations. * Statistically significantly different from data of seawater C:N=8.8 (p-value < 0.05).

3.4.7 The impact of seawater on carotenoids production of other evolved hyper-producers

Due to the complexity of metabolism, the same factor may not have same impact in different strain background. It has been reported that the addition of moderate concentrations of NaCl increased carotenoids production in some native producers (181-185), but decreased carotenoids production in other strains (149). Thus, whether the observed positive impact of seawater on carotenoids production in strain SM14 also applies to other evolved hyper-producers and the parental strain (YLH2) were determined. The carotenoids production of YLH2 and some evolved hyper-producers (SM11, SM12, SM13, SM22, SM24) were quantified in reduced nutrient condition in either fresh water or 1/3× seawater. As show in Figure 3.8, consistent with the observation with SM14, the use of 1/3× seawater improved carotenoids production of all tested evolved hyper-producers as well as the ancestor strain YLH2 compared with that in fresh water. The result suggests that the positive impact of seawater on carotenoids production is global in the engineered *Saccharomyces cerevisiae* strains.

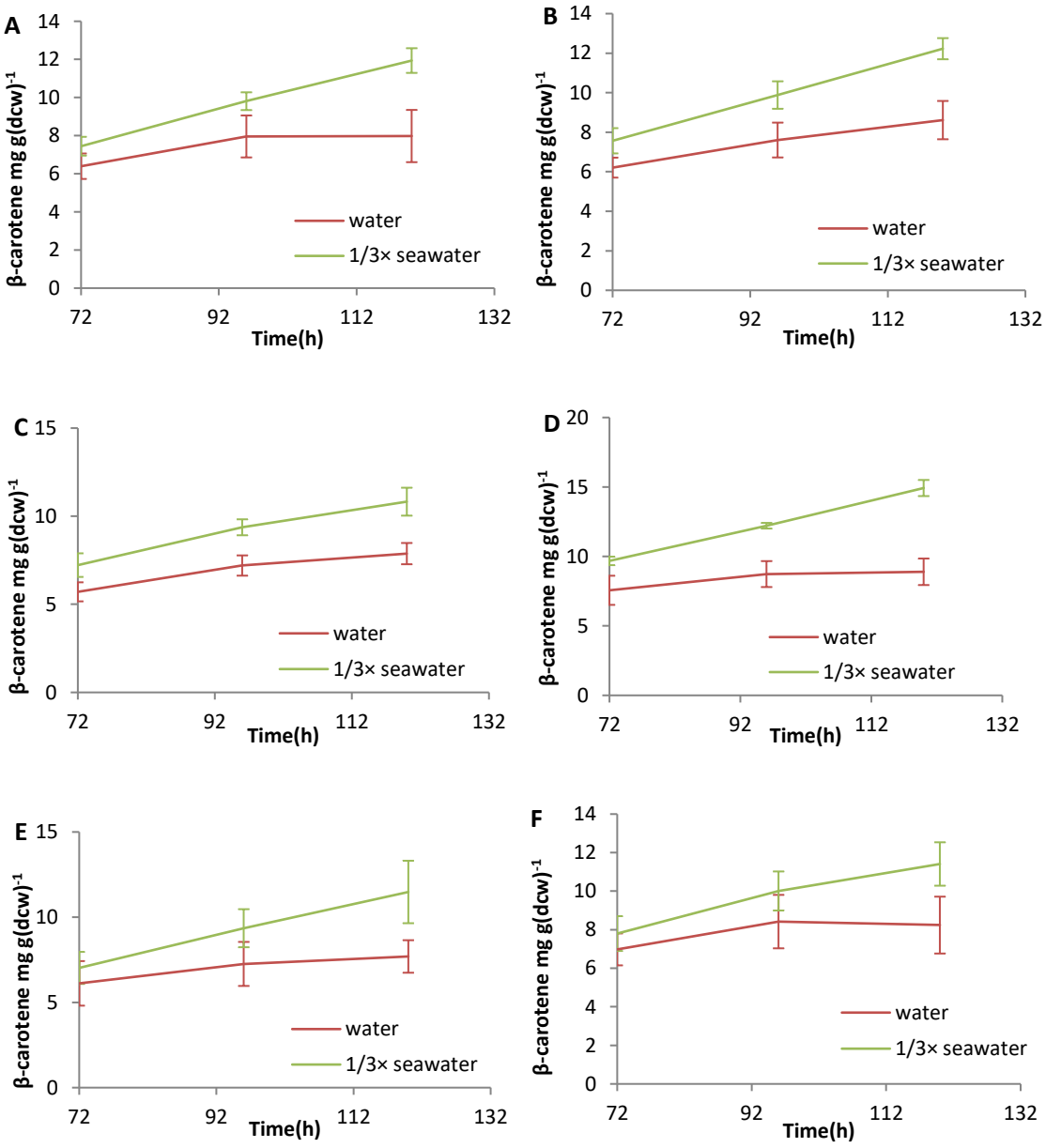

Figure 3.8. β-carotene production in 1/10× YNB made with fresh water and 1/3× seawater. (A) SM11. (B) SM12. (C) SM13. (D) SM22. (E) SM24. (F) YLH2.

3.5 Discussion

In our previous work, we found a potential correlation between fatty acid accumulation with carotenoids production in the parental strain YLH2 and the hyper-producer SM14. In this work, our results indicated that increasing C:N ratio also increased carotenoids and lipid production of SM14 in reduced nutrient conditions (Figure 3.5 and 3.6 A, B). It has been reported that salt stress affected fatty acids composition in yeast, and *S. cerevisiae* showed an increase in total sterol and a noticeable enrichment in squalene under osmotic stress (186). Squalene is involved in the mevalonate (MVA) pathway, its precursor, farnesyl diphosphate (FPP) is also the precursor of carotenoids (171). Therefore, the accumulation of squalene in *S. cerevisiae* under osmotic stress indicated that the MVA pathway was upregulated in response to osmotic stress, which might be a cause of the increased the production of carotenoids in seawater and in fresh water supplemented with additional NaCl. Our results showed that when under reduced nutrient conditions, the addition of NaCl in fresh water or using 1/3× seawater increased the fatty acid unsaturation in SM14 (Figure 3.6 C, Figure 3.7 C), which indicated that the growth in seawater may impact carotenoids production by altering lipid content of the cell. Besides the impact on lipid production, osmotic stress has been shown to trigger cross tolerance to oxidative stress (182, 187-189). Since carotenoids act as antioxidants in living cells, the higher carotenoids production in seawater might be a part of response to oxidative stress triggered by osmotic stress.

In our previous works, the hyper-producers including SM14 had significantly higher carotenoids production than their ancestor strain YLH2 in YPD and YNB media

(174, 175), however, in this work, these evolved hyper-producers did not produce more carotenoids than YLH2 under reduced nutrient conditions, which indicated that the higher carotenoids production of the evolved hyper-producers are specific to rich nutrient conditions. We have demonstrated that seawater can improve carotenoids production of SM14 under the reduced nutrient condition, but no improvement was observed when using normal YNB media (Figure 3.1). Thus, in addition to NaCl, carotenoids production is also impacted by other components in the media. The impacts of other components on carotenoids production remain to be investigated.

4 IMPROVE GROWTH OF *SACCHAROMYCES CEREVISIAE* IN SEAWATER

4.1 Summary

Using adaptive laboratory evolution, we improved the growth of *Saccharomyces cerevisiae* strain SM14 in seawater with limited nutrients. Nonsense mutations in *WHI2* were identified in the evolved mutants with improved performance in seawater through whole genome resequencing. By reconstructing the identified *WHI2* nonsense mutations in the ancestor strain SM14, we confirmed that the improved growth in synthetic seawater of the evolved mutants was conferred by the *WHI2* nonsense mutations, which also confer improved tolerance to hyperosmotic stress exerted by NaCl. Though it has been reported that the Δ*WHI2* cells were sensitive to salt stress (190-192), the *WHI2* nonsense mutations we identified and the Δ*WHI2* mutants constructed in our work did not show the same phenotype. However, these *WHI2* mutations only improved performance of SM14 under osmotic stress but not the ancestor strains YLH2 and FY2, which indicated that the response to osmotic stress of SM14 is different from its ancestor strains.

4.2 Introduction

Industrial biotechnology, which uses biological systems and renewable raw material to produce chemical, provides a more sustainable and environmentally friendly approach for chemical production. However, the treatment of renewable raw material and the fermentation process requires a large amount of water. As the population

growing, the demand of fresh water is increasing, thus, water scarcity is among the main problems to be faced by the world. According to UNDP, UN-Water and FAO, around 1.2 billion people live in areas of physical water scarcity, and 500 million people are approaching this situation (193, 194). It has been estimated that freshwater usage for biofuel production will increase by 5.5% over the water availability by 2030 (176), thus reducing the amount of fresh water used in biotechnology industry will help to alleviate water scarcity. Attempts have been made to use seawater as a replacement of fresh water for pretreatment of lignocellulosic and fermentations (195, 196). The use of seawater media for ethanol fermentation by *Saccharomyces cerevisiae* has been shown to be promising (177), and it has also been used to improve carotenoids production by *Rhodotorula glutinis* (178). However, due the high salinity of seawater (15), its adoption has not been widespread.

In this work, we evolved *Saccharomyces cerevisiae* strain SM14 in minimum media made with synthetic seawater and reduce nutrients. After about 70 generations, the performance of two separately evolved populations were significantly improved with about 24 hours shorter lag phase compared with the ancestor strain. Mutants with better performance were selected and nonsense mutations in *WHI2* were identified by genome sequencing. By reconstructing the *WHI2* mutations in unevolved SM14, we confirmed the *WHI2* nonsense mutations do confer osmotic tolerance and improved growth of SM14 in synthetic seawater, which shed light on osmotic tolerance mechanisms of *S. cerevisiae* and may promote the utilization of seawater.

4.3 Materials and methods

4.3.1 Strains and growth conditions

SM14, a hyper-producer of carotenoid was used for the adaptive evolution experiment. Strains were cultured at 30°C. Normal YNB and YPD were used for routine culture, and 1/10× YNB (0.17 g L^{-1}) made with synthetic seawater (RICCA) was used for evolution and growth essay. Strains used in this study are listed in Table 4.1.

Table 4.1 List of strains.

Name	Description	Reference
FY2	MATα *URA3*-52, isogenic to S288C	(197)
YLH2	Unevolved ancestral strain	(174)
SM14	Isolated hyper-producer from evolved population 1	(174)
P2-2	Evolved mutant from population 2 of SM14 with *WHI2* Q181X nonsense mutation	This work
P1-4	Evolved mutant from population 1 of SM14 with *WHI2* S242X nonsense mutation	This work
S-C	SM14 Δ*URA3*	This work
Y-C	YLH2 Δ*URA3*	This work
S-Δ*WHI2*::*URA3*	SM14 Δ*WHI2*::*URA3*	This work
Y-Δ*WHI2*::*URA3*	YLH2 Δ*WHI2*::*URA3*	This work
F-Δ*WHI2*::*URA3*	FY2 Δ*WHI2*::*URA3*	This work
S-Δ*WHI2*	SM14 Δ*URA3* Δ*WHI2*	This work
Y-Δ*WHI2*	YLH2 Δ*URA3* Δ*WHI2*	This work
F-Δ*WHI2*	FY2 Δ*WHI2*	This work
S-*WHI2-2*	SM14 Δ*URA3 WHI2* Q181X	This work
Y-*WHI2-2*	YLH2 Δ*URA3 WHI2* Q181X	This work
F-*WHI2-2*	FY2 *WHI2* Q181X	This work
S-*WHI2-1*	SM14 Δ*URA3 WHI2* S242X	This work
Y-*WHI2-1*	YLH2 Δ*URA3 WHI2* S242X	This work
F-*WHI2-2*	FY2 *WHI2* S242X	This work

4.3.2 Evolution experiment

Evolution experiment was conducted via serial batch transfer. Two independent populations of SM14 were inoculated from two single colonies in normal YNB and incubated at 30 °C overnight, then cells were pelleted and washed with 1/10× YNB made with synthetic seawater and inoculated into 10 ml 1/10× YNB seawater media in 125 ml flasks with initial OD_{600} ~0.05. Approximately every 48 hours, a proportion (based on cell density) of each population was pelleted and reinoculated into fresh media with initial OD_{600} ~0.05. After about 46 generations, the transfer period was shortened to 24 hours as the populations grew faster. Each population underwent about 3 generations per transfer for a total of 70 generations.

4.3.3 Mutant isolation and screening

By the end of the evolution experiment, populations were plated on 1/10× YNB seawater agar plate for single colonies and several colonies were chosen based on their bigger size. To test the stability of the evolved mutants, the colonies were grown in 3 ml normal YNB in water for about 30 generations and then transferred in 1/10× YNB seawater media for growth assay. One colony of each population with best growth performance compared with unevolved strain was selected for further analysis.

4.3.4 Genome sequencing and verification

The evolved populations, and the selected single isolates from each population, along with the unevolved parental strains, were sequenced to discover the mutations confer the observed growth improvement in seawater. Genome sequencing was performed by the Texas A&M Genomics Center for sequencing on the Illumina HiSeq

2500 platform using 100-bp single-end reads. The genome of S288C was used as reference to assemble the reads and each mutant genome was compared to the parental sequences to identify any mutations. The identified mutations were verified with Sanger sequencing.

4.3.5 Reconstruction of WHI2 mutants

The *URA3* gene of SM14 and YLH2 were excised out using 5-fluoroorotic acid (5-FOA) as counter-selection. Then the coding sequence of *WHI2* gene was knocked out using *URA3* as a marker and selected on YNB plates, and the *URA3* maker was then excised to create Δ*WHI2* strains without marker. The *WHI2* mutations were amplified from the evolved mutants and introduced into the *WHI2- URA3+* strains using 5-fluoroorotic acid (5-FOA) as counter-selection. The Frozen-EZ Yeast Transformation II Kit (Zymo Research) was used for the transformation of yeast. The reconstructed mutants were confirmed by PCR and Sanger sequencing. All primers used were listed in Table 4.2.

Table 4.2 List of primers.

Name	Sequence	Description
YIplac_f3	AACGTTGTTGCCATTGCTAC	Forward primer for homologous sequence upstream of *URA3* in SM14
YIplac_ura3_U_r1	AATACAGTTTTTCTTAGACGTCAGGTGGCAC	Reverse primer for homologous sequence upstream of *URA3* in SM14
ura3_D1_f	GACGTCTAAGAAAAACTGTATTATAAGTAAATGCATG	Forward primer for homologous sequence downstream of *URA3* in SM14
ura3_D741_r	TTGAGTGCAATCGTAGGACG	Reverse primer for homologous sequence downstream of URA3 in SM14

Table 4.2 Continued

Name	Sequence	Description
WHI2_HRL_ura3_f	CATTAATTGATAAAGATAAAGGTTGTCTGAGCTTACACTTATTATAAACACTTTCGTCTCGCGCGAAC	Forward primer for the knockout of *WHI2*
WHI2_HRR_ura3_r	GTCTTTGGCCCGATCTCTTTCCATTTCTTTCTCTAATATATTATATACACCAAAGACTCGAGCACAGG	Reverse primer for the knockout of *WHI2*
WHI2_HRL_f	CGCAAGAAGACAACTCCTTC	Forward primer for introducing mutated *WHI2*
WHI2_HRR_r	GAATAGAAAGAGGGGATACC	Reverse primer for introducing mutated *WHI2*
WHI2_357up_f	CAGCATAGGCATAGTGATAG	Forward primer for homologous sequence upstream of *WHI2*
WHI2_1up_r	ATTATATACACTGTTTATAATAAGTGTAAGC	Reverse primer for homologous sequence upstream of *WHI2*
WHI2_1down_f	TATTATAAACAGTGTATATAATATATTAGAG	Forward primer for homologous sequence downstream of *WHI2*
WHI2_448down_r	TTGCAGACTCGTGATCACAG	Reverse primer for homologous sequence downstream of *WHI2*
WHI2_Wild_451f	TGTACCTCAGGAGGAAGTTC	Wild-type primer for the verification of *WHI2* mutation in P2-2
WHI2_Mutant_451f	TGTACCTCAGGAGGAAGTTT	Mutant primer for the verification of *WHI2* mutation in P2-2
WHI2_Wild_725f	AAGGGGTCCAATTCTTATTC	Wild-type primer for the verification of *WHI2* mutation in P1-4
WHI2_Mutant_725f	AAGGGGTCCAATTCTTATTG	Mutant primer for the verification of *WHI2* mutation in P1-4
WHI2_seq_302f	GTTTTGAGTACATCATGGAG	Primer for Sanger sequencing of *WHI2*
WHI2_seq_540r	AAATTCCTCCTGAGGTACAC	Primer for Sanger sequencing of *WHI2*
WHI2_seq_876f	ATTGGCTAACGAGACAACTG	Primer for Sanger sequencing of *WHI2*
WHI2_seq_f	CGCTCCGATTCTACAAGAAC	Primer for Sanger sequencing of *WHI2*
WHI2_seq_r	CTCAAGACAACTTCATCACCC	Primer for Sanger sequencing of *WHI2*

4.4 Results

4.4.1 Adaptive evolution in 1/10× YNB made with synthetic seawater

Preliminary experiments showed that normal YNB made with seawater did not inhibit the growth of *S. cerevisiae* significantly, thus a media with reduced nutrients in seawater was used in the evolution experiment, aiming at a better performance under the condition with osmotic stress and limited nutrients. The growth of SM14 in 1/10× YNB made with fresh water and synthetic seawater was compared (Figure 4.1A), and the result showed that the using of seawater had a significant inhibition on SM14 compared with fresh water. After 70 generations evolution in 1/10× YNB seawater media, the growth kinetics of evolved populations were compared with the unevolved SM14 and both populations have similar growth kinetics, they had about 24 hours shorter growth lag and reached higher final cell density than the unevolved SM14 (Figure 4.1B). To verify the stability of the evolved mutants, selected colonies were grown in normal YNB in water for 30 generations and then inoculated in 1/10× YNB seawater media to compare their growth kinetics with the unevolved SM14. As show in Figure 4.1C, both isolates performed better than their ancestor strain SM14 and there is no significant difference between before and after 30 generations' cultivation in normal YNB, which indicated that the phenotypes of the selected mutants are stable.

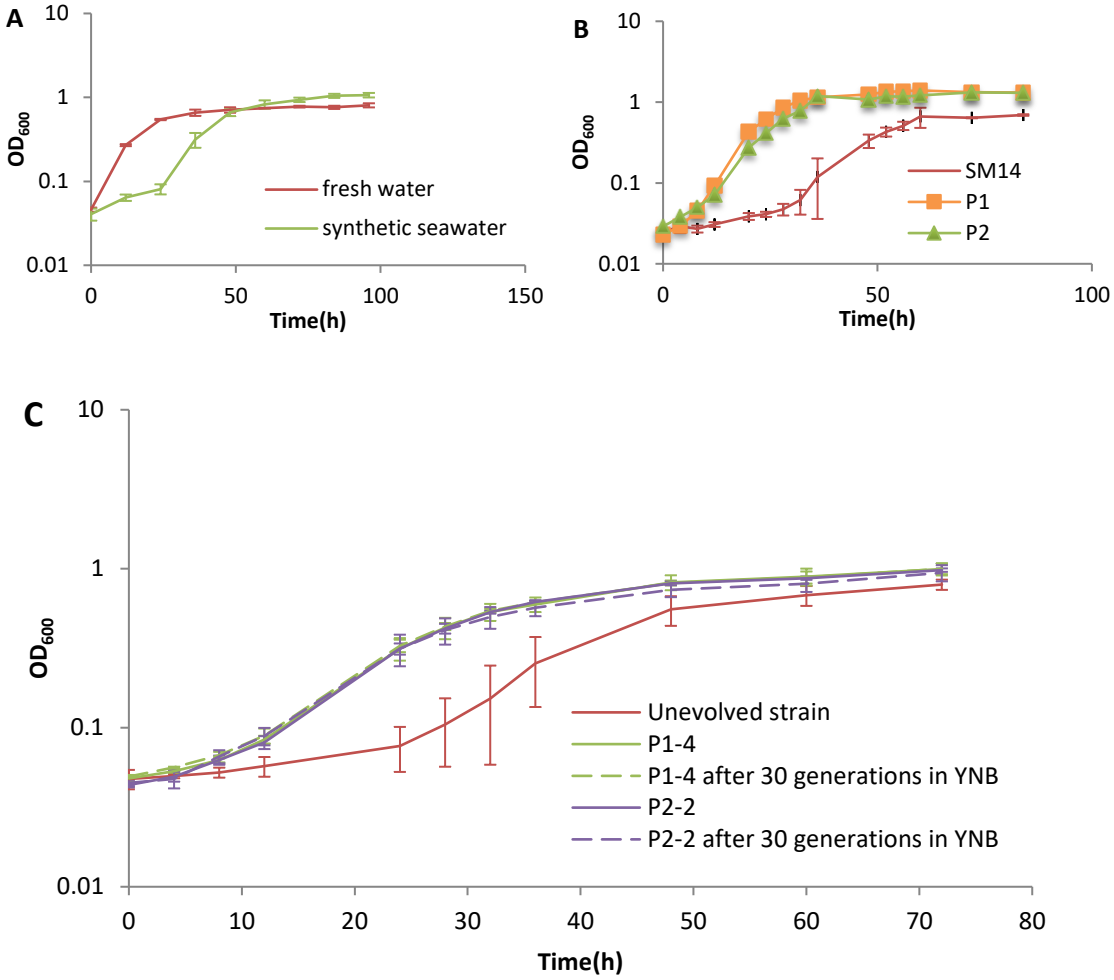

Figure 4.1. Growth kinetics. (A) Growth of SM14 in 1/10× YNB made with fresh water and synthetic seawater. (B) Growth kinetics of evolved populations and unevolved SM14 in 1/10× YNB made with synthetic seawater. (C) Growth kinetics of evolved single isolates before and after 30 generations in YNB and the unevolved SM14 in 1/10× YNB made with synthetic seawater.

4.4.2 Characterization of mutations in evolved mutants

Two isolates (P1-4, P2-2) were sequenced to detect any mutation that potentially confer the improved growth in seawater media. Nonsense mutations in *WHI2* has been identified in both isolates. The mutation in P1-4 is *WHI2* S242X, and the mutation is P2-

2 is *WHI2* Q181X. The *WHI2* Q181X exist in population 2 with a frequency of 74.43%, while *WHI2* S242X exist in population 1 with a frequency of 2.60%. Both mutations and a knockout mutation of *WHI2* were constructed in the unevolved SM14, its ancestor strain YLH2, and their wild-type strain FY2. The growth kinetics of constructed mutants were compare with their wild-type strains in seawater media (Figure 4.2). Similar as the evolved mutants, the reconstructed mutants with the nonsense *WHI2* mutations and the Δ*WHI2* mutant of SM14 had significantly shorter lag phase than the unevolved SM14 in 1/10× YNB media made with synthetic seawater. However, these *WHI2* mutations did not improve the performance of YLH2 or FY2 in seawater.

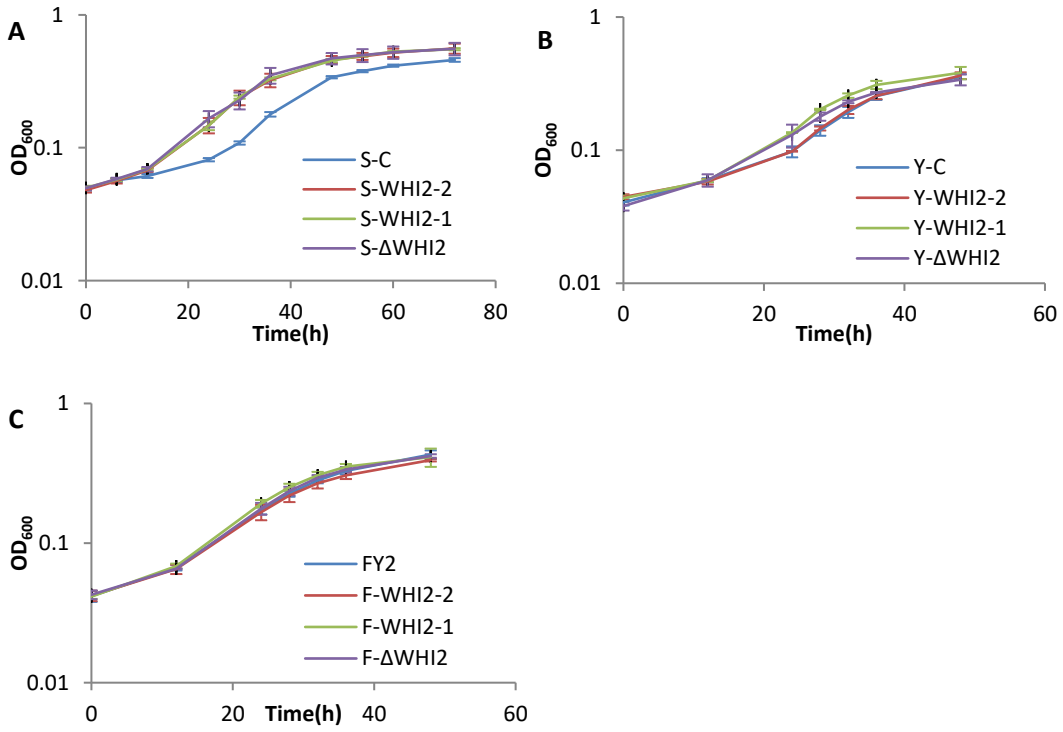

Figure 4.2. Growth kinetics of *WHI2* mutants in 1/10× YNB media made with synthetic seawater. (A) SM14 (B) YLH2 (C) FY2.

Since the main inhibitor in seawater is the high content of NaCl (0.41 M), we hypothesized that the improved growth conferred by the *WHI2* mutations in seawater is a consequence of improved osmotic tolerance. Thus, we compare the growth of the reconstructed mutants in 1/10× YNB media in fresh water with supplementation of 0.41 M NaCl. As show in Figure 4.3, both reconstructed *WHI2* mutants and the Δ*WHI2* mutant of SM14 showed better performance under osmotic stress compared with the wild-type *WHI2* strain.

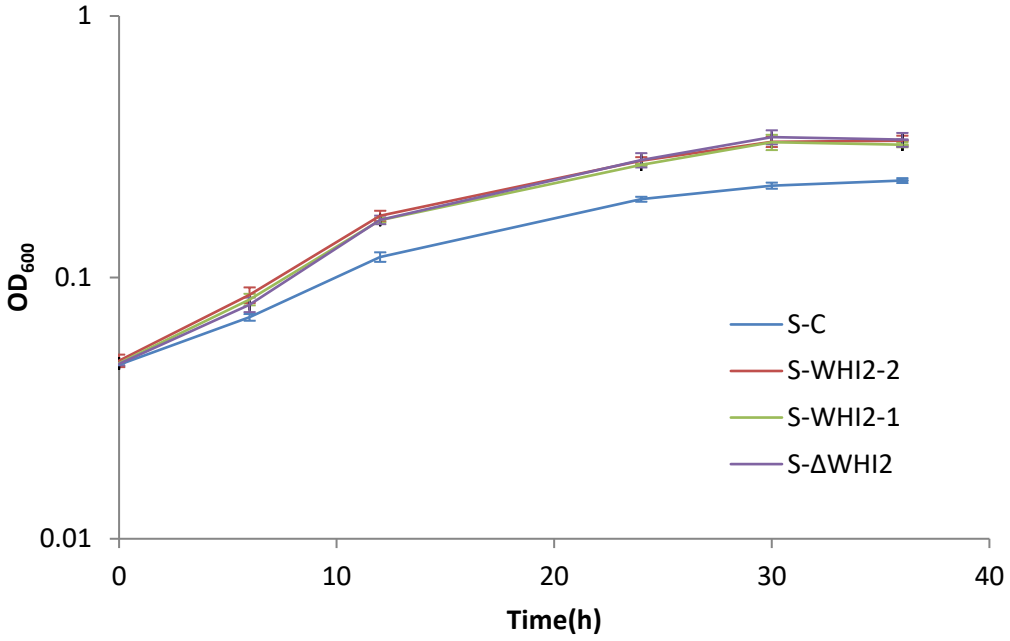

Figure 4.3. Growth kinetics in 1/10× YNB media made with fresh water with supplementation of 0.41 M NaCl.

4.5 Discussion

Whi2 is known to play essential roles on cell proliferation and general stress response. Whi2 is a negative regulator of CLN1/2 (encode G1 cyclin) expression that couples nutrient availability to cell division (190). It has been reported that the Δ*WHI2* cells exhibited abnormal growth and proliferation upon carbon exhaustion (198-201), and were sensitive to heat and salt stress (190-192). However, in our work, the evolved *WHI2* mutants and the Δ*WHI2* mutants improved growth of SM14 in seawater and under osmotic stress with 0.41 M NaCl. Though the mutants in our work did not improve performance of YLH2 or FY2, they do not show higher sensitivity to the osmotic stress generated by seawater. This is not too surprising since it has been reported the Δ*WHI2* mutation in KA31-2A background of *S. cerevisiae* also did not show growth defect under osmotic stress, which indicating that the sensitivity to osmotic stress of Δ*WHI2* mutants are dependent on strain background. Since the *WHI2* mutations only improved osmotic tolerance of SM14 but not YLH2 or FY2, there might be mutations in SM14 that alter the response of SM14 to osmotic stress. By investigating the difference of response to osmotic stress between SM14 and YLH2 or FY2, more knowledge about the mechanisms of osmotic tolerance in *S. cerevisiae* could be gained.

5 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

In this dissertation, the osmotic tolerance conferred by a *rpoC* K370_A396dup mutation in *E. coli* strain BW25113 has been confirmed and the mechanisms have been identified by a combination of several analysis methods. From individual amino acid supplementation studies, we found several additional amino acids (*e.g.* tryptophan, phenylalanine) to play a role in improving osmotic tolerance in *E. coli*. Results from metabolite analysis suggesting alterations in metabolism may be partially responsible for the enhanced hyperosmotic tolerance in the mutant. Membrane damage analysis using PI staining showed a lower effect on membrane integrity in the presence of hyperosmotic stress in the *rpoC* mutant in exponentially growing cells. Transcriptional analysis demonstrated that the *rpoC* mutation impacted transcription of genes related with amino acids metabolism, including *metK* and *mmuP*, when overexpressed in BW25113, conferred enhanced osmotic tolerance, indicating that the alternation of methionine metabolism is involved in enhanced hyperosmotic tolerance in the *rpoC* mutant.

We also demonstrated the usage of seawater water to improve carotenoids production of the engineered hyper-producer SM14. Subsequent analysis revealed that the improvement of carotenoids production by using seawater is partly due to the additional NaCl in seawater, which alters lipid content and composition. We also showed that the impact of seawater on carotenoids production also applied to other engineered *S. cerevisiae* carotenoids producers.

Using adaptive laboratory evolution, we have successfully improved the growth of *S. cerevisiae* strain SM14 in seawater with limited nutrients, and the *WHI2* nonsense mutations have been identified. By reconstructing the *WHI2* mutations in wild-type strains, we confirmed that the improved growth in seawater was due to the improved osmotic tolerance conferred by the *WHI2* mutations.

These results provide insights on the mechanisms of osmotic tolerance. They will be helpful for the rational development of robust strains for biotechnological industry, and for the expanding of the utilization of high saline feedstocks and water sources.

5.2 Recommendations for future research

RpoC is a subunit of the RNA polymerase complex, thus the *rpoC* mutation is expected to have global impact on the transcriptional regulation of the cell. In addition to osmotic tolerance, the *rpoC* mutation may also confer other phenotypes. It has been reported that other *rpoC* mutations in *E. coli* improved tolerance to low pH (202), improved growth on glycerol (203), and reduced tolerance to high temperature (204). Thus, this *rpoC* mutation can be used for the identification of mechanisms of other phenotypes in *E. coli*.

In this dissertation, the impact of seawater on carotenoids production was studied in batch cultures. In order to expand the utilization of seawater in industry, the reaction could be scaled up in bioreactors. Since there are multiple factors affecting carotenoids production, the effects of the combination of seawater with other factors and feedstocks could be investigated for the optimized condition to produce carotenoids in industry.

Since the *WHI2* mutations have different impacts on SM14 and its ancestor strains YLH2 and FY2, there should be differences on response to osmotic stress between SM14 and YLH2 or FY2. Thus, by comparing the response to osmotic stress between SM14 and YLH2 or FY2, more knowledge can be gained about the mechanisms of osmotic tolerance and the improved growth in seawater conferred by the identified *WHI2* mutations in *S. cerevisiae*.

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